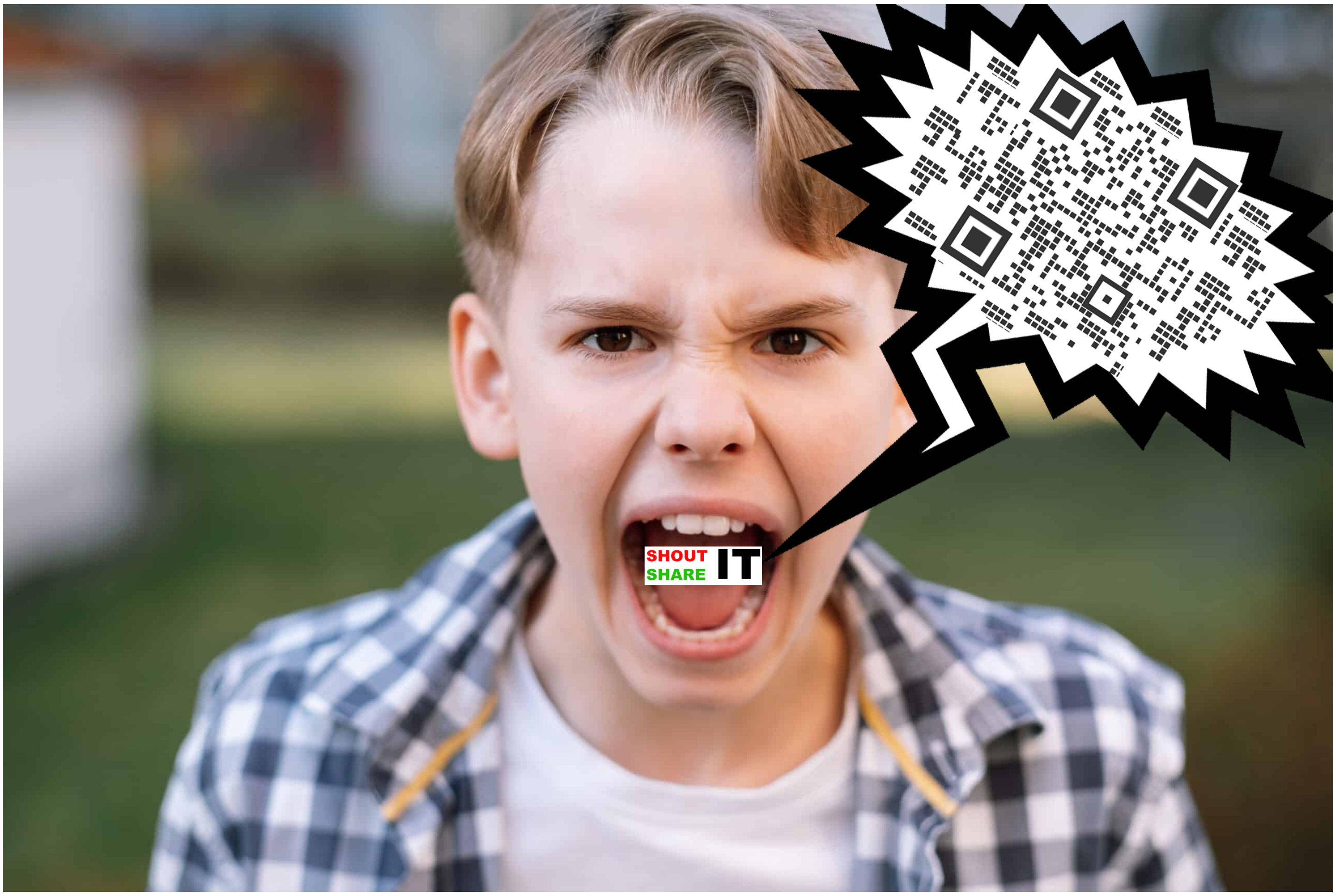
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AUTHOR'S ERRATUM

Reference: Scheer, Bradley T. The hormonal control of metabolism in crustaceans. IX. *Biol. Bull.*, **116**: 175–183 (February, 1959).

Table III on page 179 should be Table IV; Table III was inadvertently omitted. The mean total carbohydrate of the blood for 15 animals was 10.3 mg. per 100 ml., with a range of 5.8 to 15.9. There were no differences between normal and eyestalkless animals in Stage C_4 or D_1.

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THE

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THE INFLUENCE OF PRESSURE, TEMPERATURE AND URETHANE ON THE LUMINESCENT FLASH OF MNEMIOPSIS LEIDYI[1]

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Marine Biological Laboratory, Woods Hole, Mass., Department of Biology, Brown University, Providence, R. I., and Department of Biology, Princeton University, Princeton, N. J.

The luminescent flash response, induced by electrical stimulation of small segments of excised meridional canals of *Mnemiopsis leidyi,* has been recently analyzed in relation to temperature and certain other factors (Chang, 1954). This flash, as well as that of the firefly (Chang, 1956), has been found to resemble, in important respects, the contraction response of directly stimulated muscle fibers. Temperature relations of muscular contraction and various other biological processes, including specific enzyme action, bacterial luminescence, cell division, nerve activity, etc., are subject to modification by increased hydrostatic pressure. Moreover, temperature-pressure relations may be influenced by the presence of narcotics such as alcohol or urethane as well as other chemical agents (*cf.*, Johnson, Eyring and Polissar, 1954; Johnson, 1957; Brown, 1957; Marsland, 1957; Tasaki and Spyropoulos, 1957; Spyropoulos, 1957a, 1957b).

Since studies of pressure-temperature-inhibitor relations have proved a useful approach to understanding certain aspects of the chemical and physiological control of biological processes, and since studies incorporating all three variables are yet available with respect to relatively few processes, the present investigation of the *Mnemiopsis* flash was undertaken. Unfortunately, no separate biochemical components of the luminescent system have been obtained thus far from this organism, and it does not secrete a luminous slime, so the pressure-temperature relations could not be studied in regard to the luminescence of both whole organs and the reaction system *in vitro*, as was recently done with *Chaetopterus* (Sie, Chang and Johnson, 1958). More than 8000 individual flashes of the excised *Mnemiopsis* organs, however, have been accurately measured and carefully analyzed to constitute the basis of this study.

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[2] Present address: National Institutes of Health, Bethesda, Maryland.

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Materials and Methods

Mnemiopsis leidyi collected around Woods Hole, Massachusetts, and kept in large aquaria with very slowly running sea water for not more than two days, were used for this study. As previously shown (Chang, 1954), reproducible responses to electrical stimulation are obtained only with small portions of the photogenic organs, which are closely associated with the meridional canals. For experiments, the canals, with their closely adjacent tissues, were carefully excised. A small piece, measuring from 1.5 to 4 mm. in length, and including from one to four paddle plates, was cut out for the test material. This piece was then placed in a lucite chamber which in turn was sealed in a pressure bomb with a glass window as previously described (Sie, Chang and Johnson, 1958) for the purpose of stimulation at normal or under increased hydrostatic pressures.

A pair of Ag-AgCl electrodes in the specimen chamber was connected to an electronic stimulator which had controllable parameters of pulse amplitude, duration, repetition frequency and synchronization delay. The flash response was recorded by means of a stabilized photomultiplier-amplifier unit described by Chang (1954). The two beams of a dual-beam cathode-ray oscillograph were fed respectively by the output of the light detection unit and by the stimulus signal, and were photographed on a continuously moving film or with a single-frame camera.

Increased pressure was applied by means of an oil-filled hydraulic pump operated by hand. Pressures up to 10,000 pounds per square inch (psi) could be applied within approximately one second.

Results

The time course of the flash response

The time course of luminescent intensity in the *Mnemiopsis* flash has been shown to remain unaltered with increasing flash maxima due to increasing strength of stimulation (Chang, 1954). Results obtained in the present study show that, with a given strength of stimulation, increased pressure reduces the flash maxima but the time course of intensity again remains essentially the same. Figure 1 illustrates superimposed tracings of oscilloscope records from a single specimen under different pressures up to 1000 psi at room temperature. With this specimen, higher pressures diminished the flash intensity so much that the form of the response was hardly analyzable.

Temperature has a marked effect on the time course of the responding flash, which becomes progressively prolonged as the temperature is lowered (Chang, 1954). At a given constant temperature, between 35 and 15.5° C., however, the time course was found to remain unaltered by increased pressure.

Latent period

According to a limited amount of data obtained in the present study with respect to the latent period between the time of stimulation and the onset of luminescent response, no significant variation was induced by pressure. While a critical study of this relationship would require additional experiments specifically designed for this purpose, it appears likely that the differences in the latent period under normal and increased pressures would be quite small, if any.

Pressure versus flash height at constant temperature

The initial effect of increased pressure was always to reduce the intensity of the flash, and remarkably small amounts of pressure were required to produce a detectable decrease in flash height, so small in fact that they could not be read accurately on the hydraulic pump's gauge, which was not calibrated for pressures less than 200 psi. Moreover, when applied suddenly, as little as 1000 to 1500 psi often caused a virtually complete inhibition of the luminescent response (Fig. 2, A and C). Frequently, though not invariably, however, a process of adaptation under a sustained pressure took place, whereby during continued stimulation at a given frequency the flash reappeared and facilitated to successively higher maxima, sometimes reaching intensities several times greater than the highest intensity observed with identical stimuli prior to compression (Fig. 2, C). Apparently, this same

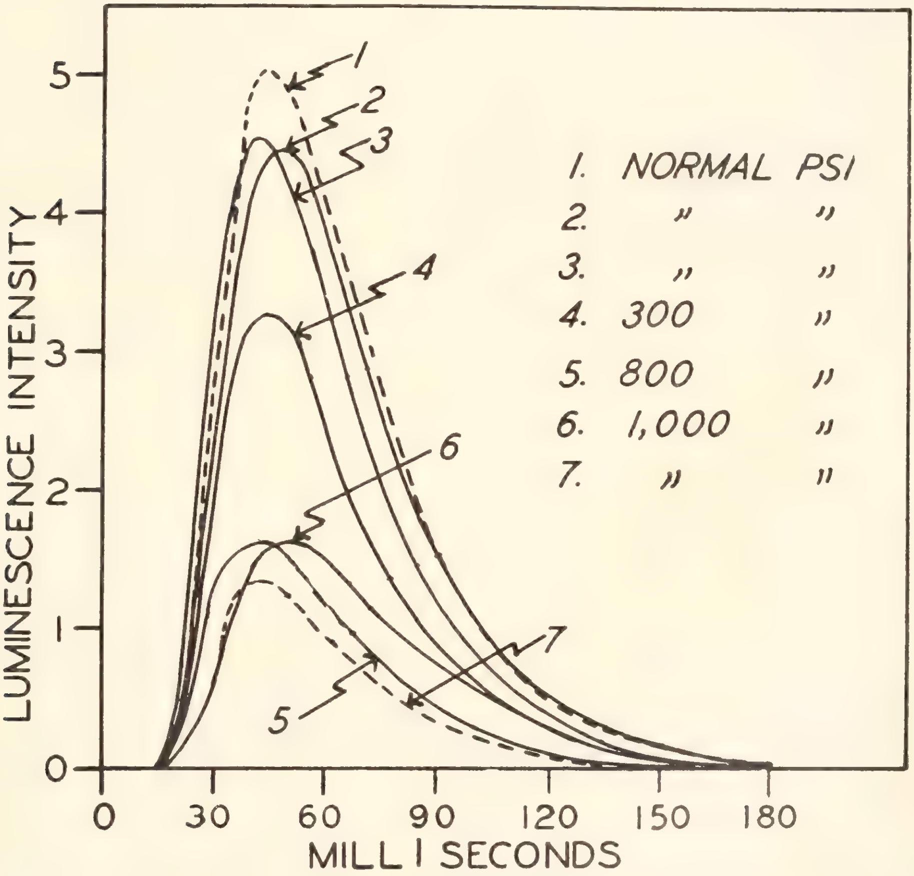

FIGURE 1. Superimposed tracings of oscillograph records of luminescent responses of a single specimen at 22° C., under normal and various increased pressures, applied in a step-wise series. The time was measured from the front edge of the square pulse used for stimulation.

process of adaptation occurred to various extents during step-wise application of pressure in small increments, inasmuch as such step-wise increases up to a given pressure were considerably less inhibitory than a sudden increase to that pressure (Fig. 2).

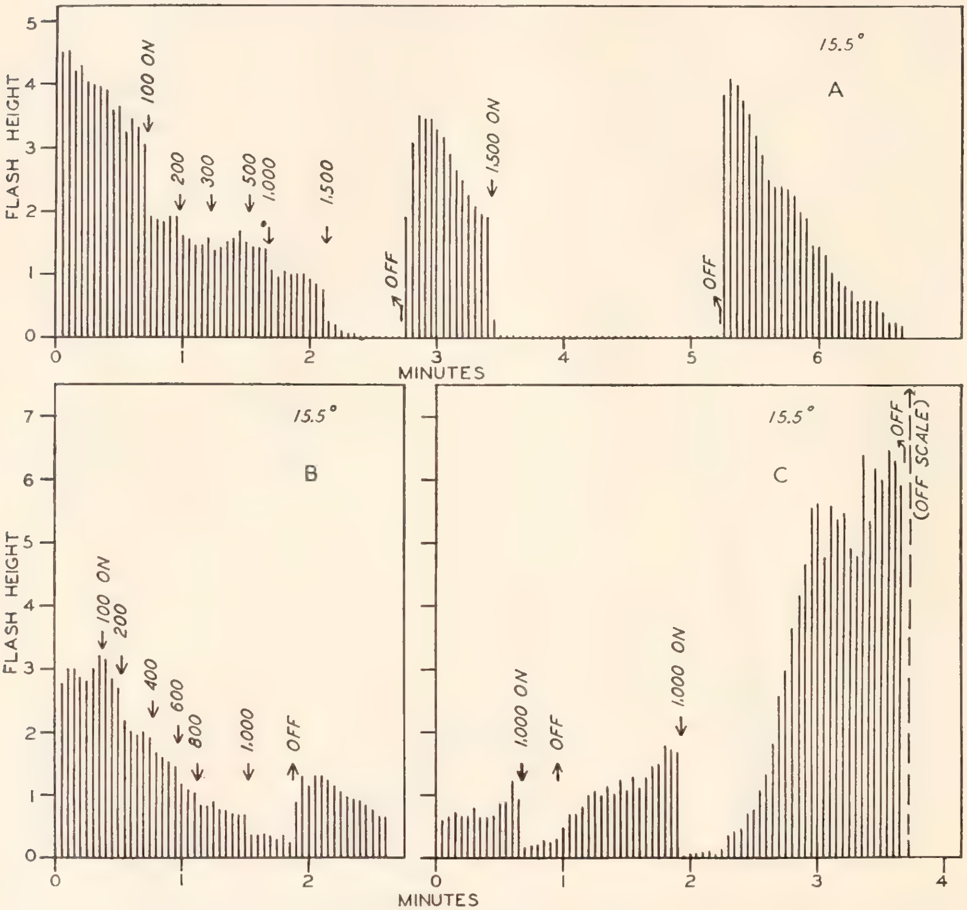

FIGURE 2. Intensities of flashes responding to repeated, identical rectangular pulses at the rate of one every 3 seconds. B and C were taken from the same specimen, and A from another. The downward arrows represent the time of application of the various pressures indicated in psi, and upward arrows represent decompression to normal pressure. The flashes that went off scale in C reached a height of 16 or above on the relative scale of the figure when measured at a lower sensitivity of the phototube.

The initial effect of decompression was essentially always an increase in flash maxima over those occurring while under pressure, or in some instances those occurring prior to compression (Fig. 2, A, B, C). Such increases sometimes attained dramatic proportions, especially in those instances wherein adaptation under pressure had taken place to a very marked extent, as indicated in Figure 2, C and

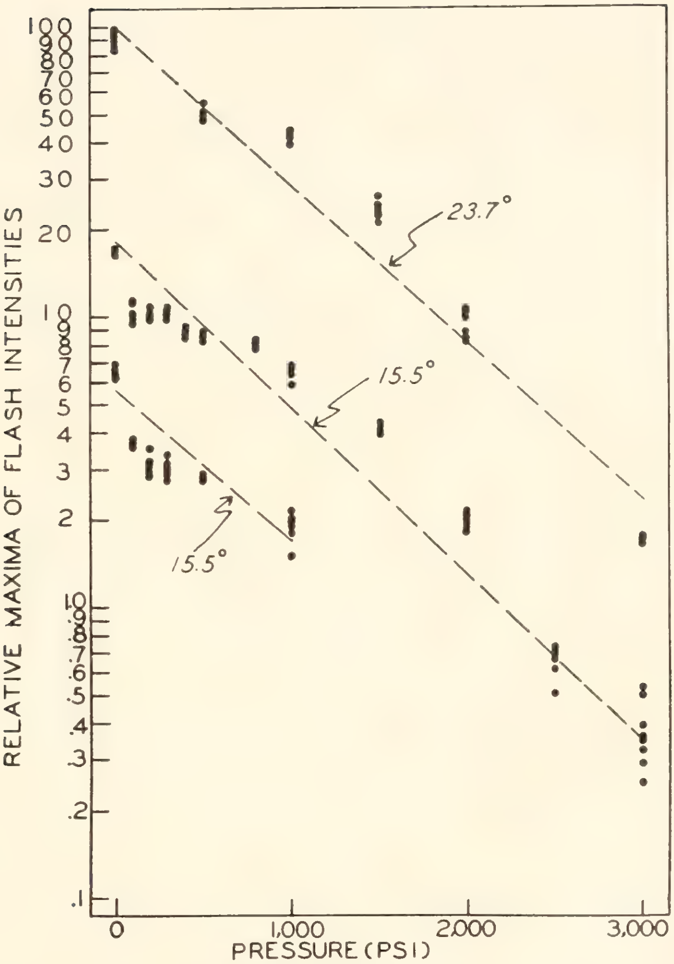

FIGURE 3. Relation between pressure and logarithm of flash intensity, in a step-wise series of pressure increases during repetitive responses to identical stimuli. Dashed lines were drawn by inspection, the lower two lines pertaining to a specimen in sea water, and the uppermost line to a specimen in sea water containing 0.1 *M* urethane.

illustrated more clearly in later figures. The only exceptions to increasing flash maxima following decompression occurred when, for unknown reasons, the specimen deteriorated under pressure with complete loss of excitability (Fig. 6, A).

Because of a wide variability in the quantitative effects of pressure on different specimens, and the phenomenon of adaptation that occurred to various extents at unpredictable rates, reliable data concerning the relation between amount of pressure and of effect produced are obviously difficult to achieve. The physiological state of the specimen at the moment of the experiment was evidently an important factor in the results obtained. The most feasible approach to investigating the quantitative relation between amount of pressure and effect produced appeared to be through a series of rapid, step-wise pressure increases, that would permit a minimum of adaptation in a given specimen, under repetitive stimulation by square pulses of identical voltage and duration fired at a constant frequency. The results of such a series at 15.5° C. are shown in Figure 2, A and 2, B. Although analysis of these results is subject to the complicating factors referred to above, data from Figure 2 and from two other experiments are plotted in an analytical manner (*cf.*, Johnson, Eyring and Polissar, 1954) in Figure 3, where each point represents the height of an individual flash in a series of three to eight flashes immediately before or after a change in pressure, at a constant stimulation frequency of one every three seconds throughout.

Despite the numerous factors that potentially influence the observed results, the relationship between the logarithm of relative flash height and the amount of pressure under which the response occurred appears to be roughly linear. The slopes of the dashed lines drawn by inspection in Figure 3 indicate a molecular volume change of about 170 cc. per mole for the over-all process.

Pressure effects at different temperatures

At a temperature as low as 5° C., strong stimuli elicited only a weak response at normal pressure. Under 1000 psi the response was abolished and it failed to return after decompression, so further experiments at temperatures this low were abandoned. A large number of experiments were done within the range 15 to 36° C., however, and representative results are illustrated in Figures 4, 5, and 6, in addition to Figure 2.

Qualitatively, no pronounced differences in the effects of pressure at the different temperatures were found. The same phenomena, and same sort of variability as described above for experiments at 15.5°, were encountered at all the higher temperatures studied. Quantitative differences are difficult to make certain of, for the reasons already indicated. Certain generalizations, however, may be adduced from the data, as follows.

First, at all temperatures the initial effect of pressure was to reduce the intensity of the flash.

Second, at all temperatures a sudden compression was more effective in reducing the flash intensity than was a more gradual or step-wise increase in pressure.

Third, adaptation and facilitation under pressure varied unaccountably. Out of the total number of experiments performed, they failed to occur in a larger number of instances than they did occur. In some instances they failed to occur during reasonably long periods of sustained pressure (Figs. 2, A; 4, A; 5, B; 6, A) even though excitability was not destroyed, as shown by recovery after decompression. In other instances, they occurred readily, sometimes resulting in flash intensities greatly exceeding those at normal pressure as already noted (Fig. 2, C, 1000 psi), or at pressures as high as 3000 psi (Fig. 5, A), 4000 psi (Fig. 6, B) and 5000 psi (not illustrated). Moreover, adaptation and facilitation sometimes occurred promptly on raising the pressure from a given high pressure, where they had not appreciably occurred, to a still higher pressure, *e.g.*, after raising from 2000 psi to 3000 psi (Figs. 4, A and 6, B).

Fourth, although sudden decompression always led to an increase in flash intensity, the pattern of changing maxima in successive flashes varied considerably.

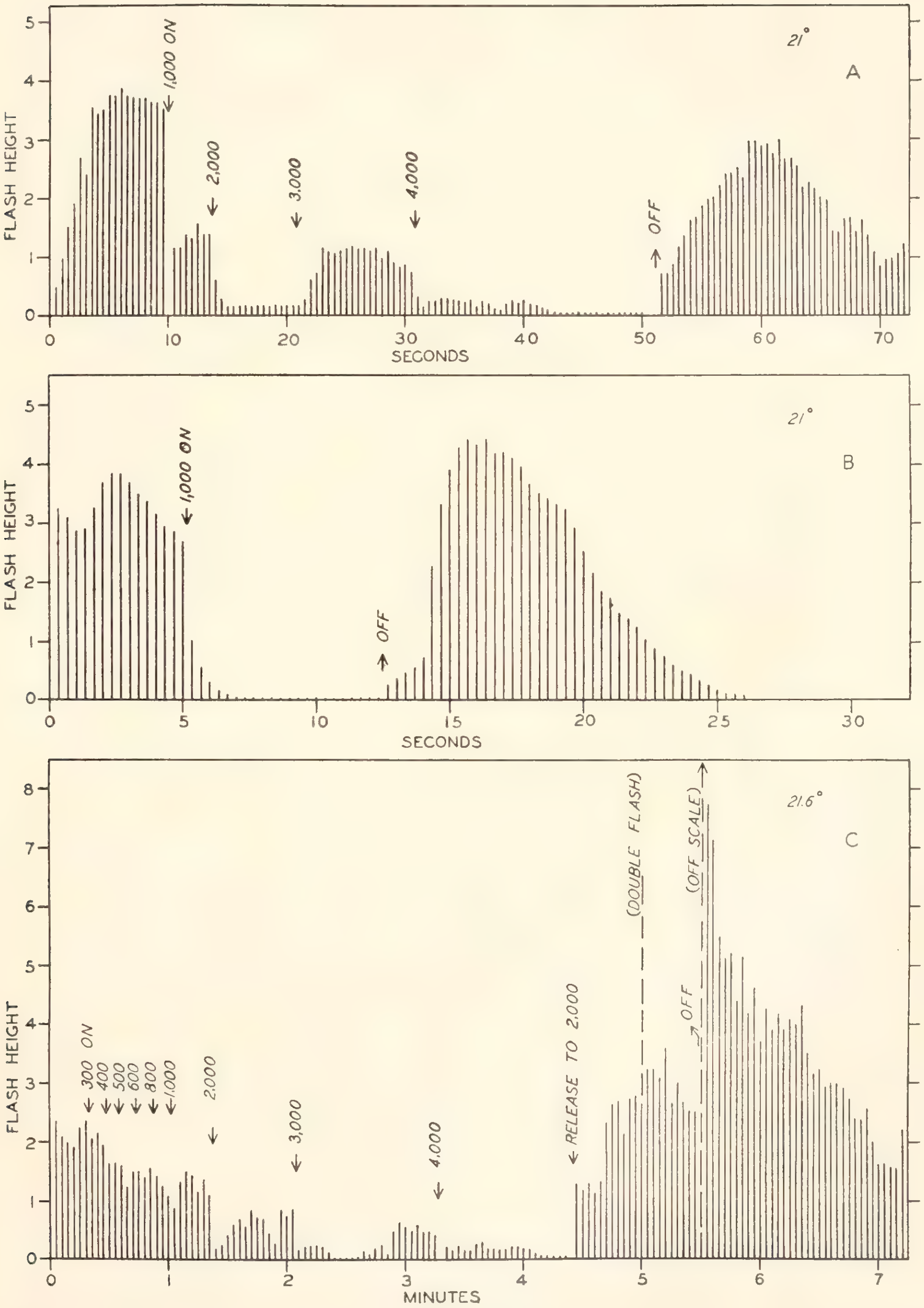

FIGURE 4. Intensities of flash response to repeated identical stimuli, at the rate of one every 3 seconds (A and C) and of 3 per second (B). Arrows represent the time of applying or of releasing, pressure indicated as psi. Room temperature.

In some instances there was a relatively large "overshoot" in the first one or two flashes after decompression, followed by a fairly rapid decline (Figs. 2, A; 4, C; 5, A; 5, B; 6, A), whereas in other instances decompression was followed by a more or less gradual facilitation (Fig. 2, C and Fig. 4, B, after 1000 psi; Fig. 4, A, after 4000 psi). Out of the total series of experiments, a very few specimens deteriorated and failed to recover at all (*e.g.*, Fig. 6, A, after 5000 psi).

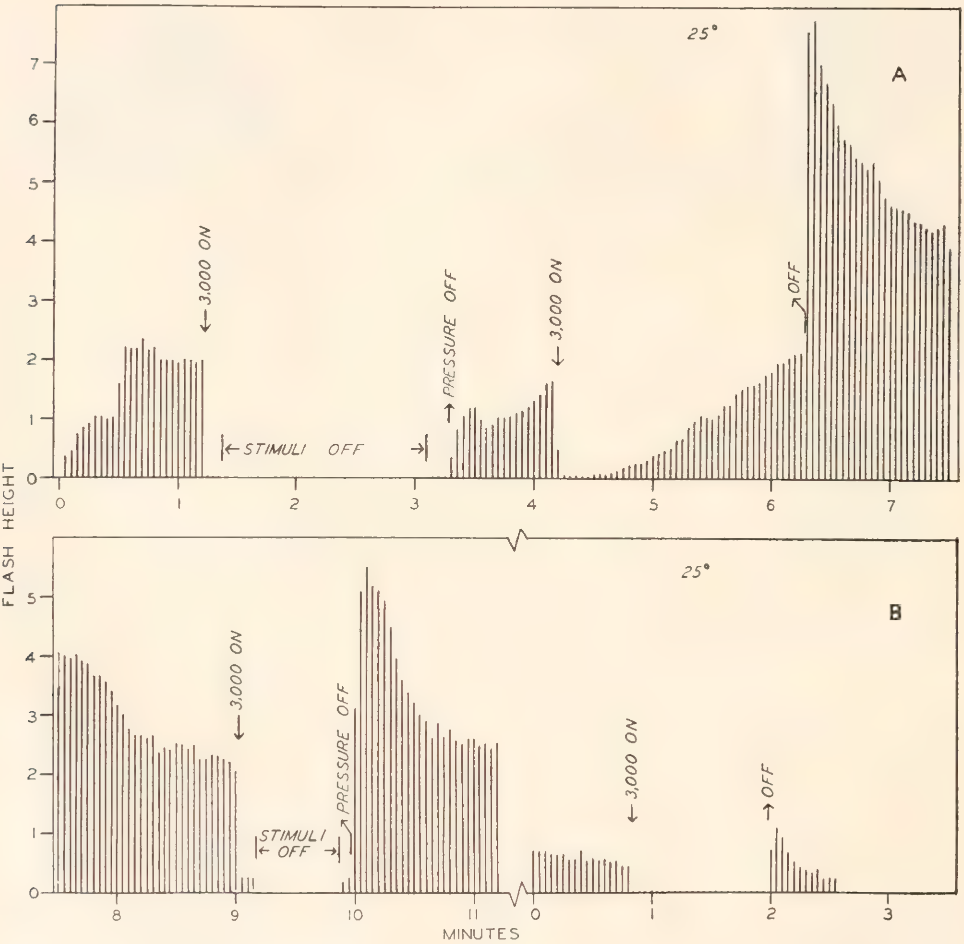

FIGURE 5. Influence of pressure on flash responses, at 25° C., to repetitive stimulation at a frequency of one every three seconds. During two periods under pressure, stimulation was discontinued as indicated in the figure. The complete series, A and B, is from a single specimen, with a rest period indicated by the break in the abscissa of B.

Fifth, excess flash intensities after decompression were not dependent on maintaining repetitive stimulation during the period of sustained pressure (Fig. 5, A and B).

Sixth, qualitatively the same phenomena were observed when a high as well as when a low frequency of stimulation and response were involved. A representative example of a high frequency of stimulation, *i.e.*, 3 per second, is shown in Figure 4, B, for comparison with the more commonly employed frequency of one every three seconds. The higher frequency was inconvenient as a routine, both because of the rapid fatigue always associated with it, and the difficulty of applying a desired pressure in a fraction of a second between flashes.

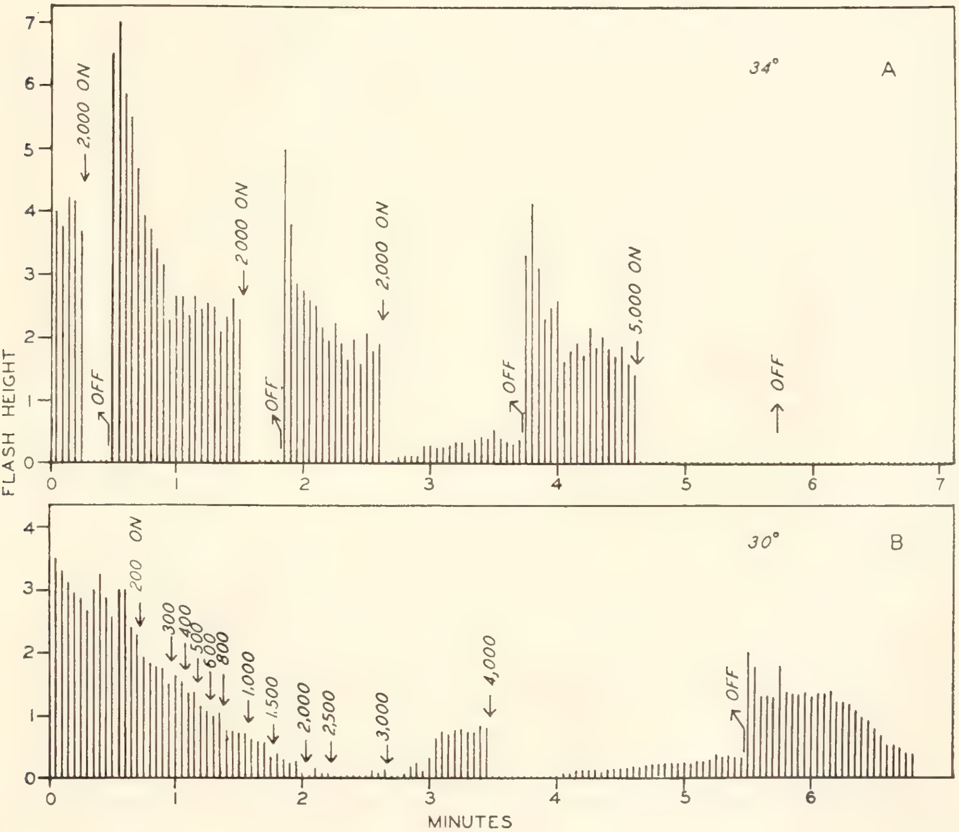

FIGURE 6. Flash responses under various pressures, at 34° (A) and 30° C. (B), at a stimulation frequency of one every three seconds. Two different specimens.

Finally, with reference to adaptation and facilitation under pressure, as well as to excess flash intensities following release of pressure, a noteworthy observation was made on a number of occasions, namely, that specimens which had lost their excitability, through fatigue or other causes, could be rendered excitable again merely by holding them under 3000 to 5000 psi for periods of one to five minutes. Such treatments were not invariably successful, of course, inasmuch as deterioration beyond the possibility of recovery sometimes occurred.

Urethane and pressure

At room temperature, 1.0 M urethane in sea water quickly abolished the luminescent response. Lower concentrations of 0.5 down to 0.05 M caused inhibitions that varied in extent with the individual specimen, the amount of adherent jelly, and duration of exposure to the drug. Although some specimens gave luminescent flashes, at reduced intensity, in 0.5 M urethane, at least for a short period of time, other specimens very rapidly lost their excitability in 0.25 M. In 0.2 to 0.15 M urethane the response of excised canals disappeared after a few minutes and the cilia stopped beating, although in sea water without urethane the luminescence and ciliary action would often persist for a couple of days. In 0.1 M urethane the response was lost after about half an hour, but in 0.05 M it persisted dimly for a longer time. Thus, urethane causes a progressively increasing inhibition of the flash response, at a rate depending on the concentration added, being faster the greater the concentration. In studying the influence of pressure, therefore, the experiments were carried out within a short period of time after adding the drug.

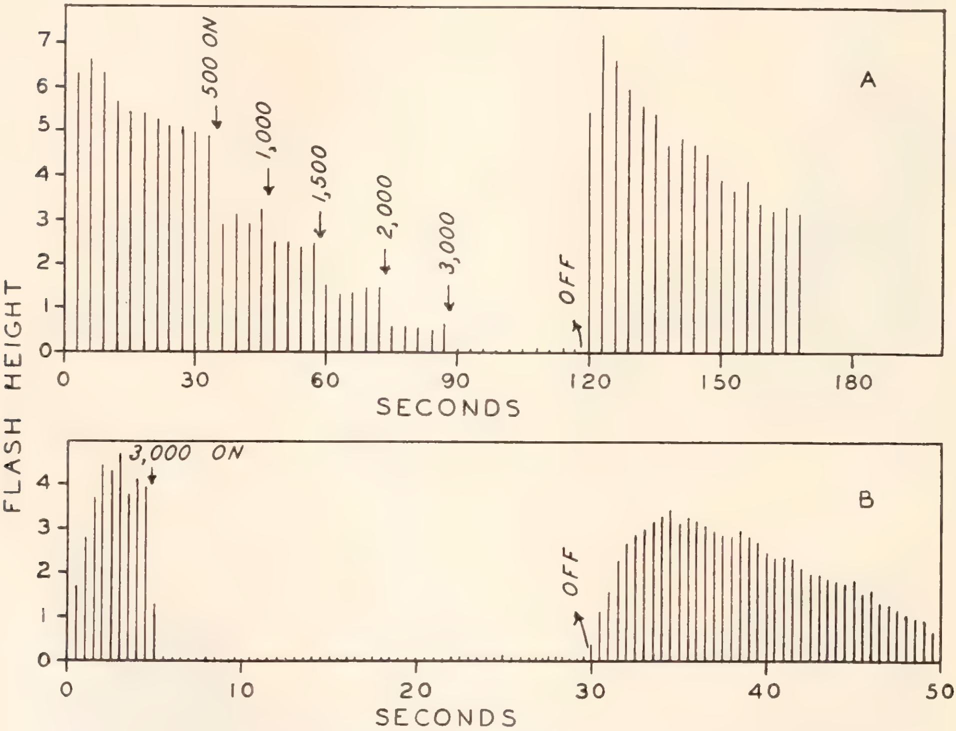

FIGURE 7. Flash responses, under various pressures, of a single specimen in sea water containing 0.1 M urethane at 23.7° C. Stimulation frequencies were one every 3 seconds (A) and 2 per second (B).

The results showed that the generalizations described in the preceding section with respect to the action of pressure in absence of urethane are qualitatively applicable in the presence of urethane. Representative data are illustrated in Figure 7, for 0.1 *M* urethane and two different frequencies of stimulation. The instability of the system in the presence of urethane, especially in the higher concentrations or at higher temperatures, made satisfactory experiments difficult to carry out, and the quantitative significance of the results uncertain. The data indicate, however, that no marked difference in volume change for the over-all process of the flash is caused by urethane (see Fig. 3).

Discussion

The *Mnemiopsis* flash is obviously limited by two types of processes, namely, physiological excitation that leads to a luminescent response, and biochemical reactions involved in light emission itself. While detailed information is not available with respect to either of these processes, it is reasonable to believe that pressure can alter the response by influencing the state of the activating system in the photogenic organ just prior to, or at the time of stimulation, as it does in muscle (Brown, 1957). Moreover, it may be assumed with considerable assurance that the process of light emission is limited by the activity of one or more essential enzymes. The probability that *Mnemiopsis* luminescence is directly dependent on more than one enzyme is suggested by the somewhat complicated effects of pressure described in this paper, as well as by the inability to demonstrate a simple "luciferin-luciferase reaction," *i.e.,* light emission on mixing a boiled and cooled aqueous extract of the triturated photogenic organs with a cold-water extract of similar organs. Even with a single limiting enzyme, pressure may affect the over-all process through several mechanisms. Two mechanisms of potential importance are (1), by influencing an equilibrium between catalytically active and reversibly inactivated states of the enzyme, the actual state depending upon temperature and the conditions of the chemical environment such as presence of drugs, ions, or other agents that act on the equilibrium, and (2), by influencing the catalytic process itself, *i.e.*, the change from normal to activated states of the reactants. Where consecutive reactions are responsible for the measured results, the effects of increased pressure are liable to become considerably more complicated, and transitory changes from one steady-state to another can assume a variety of patterns, such as an initial augmentation followed by an inhibition, or vice versa, with a converse pattern associated with the release of pressure.

In the present experiments the immediate effects of changes in pressure are attributable to an immediate change in one or more specific reaction rate constants, or possibly equilibrium constants. The phenomena of adaptation and facilitation under pressure, as well as "overshoot" following decompression, are indicative of changes in steady state concentrations of reactants, although effects on slowly changing states of equilibria cannot be ruled out. Such equilibrium changes could pertain, *a priori*, either to the process of physiological excitation or to enzymes involved in light emission, but the fact that "overshoot" and excess luminescence following a period under pressure are not contingent on continued stimulation during that period argues against the process of physiological excitation as the major site of action.

The pronounced instability of the *Mnemiopsis* system at high temperatures or in the presence of urethane makes it unusually difficult to find evidence of any reversible, inactivation equilibrium change, analogous to those which limit other processes that undergo less rapid destruction under the influence of these factors (Johnson, Eyring and Polissar, 1954). In *Mnemiopsis,* if such equilibria exist, they are obscured by the essentially irreversible processes, and the effect of pressure on these equilibria becomes correspondingly difficult to detect.

Finally, some basic similarities should be pointed out with respect to the influence of pressure on the electrically stimulated flash in excised segments of the *Mnemiopsis* organ, and on the much slower flash in excised notopodia of *Chaetopterus* (Sie, Chang and Johnson, 1958). Thus, both exhibit initial reductions in flash maxima, followed by adaptation and facilitation under pressure, as well as "overshoot" and excess luminescence on release of pressure. Parenthetically, in view of the fact that the vertical distribution of certain ctenophores extends to depths of some 2500 meters (Chun, 1903), where the pressure amounts to 250 atmospheres or about 3500 psi, it is interesting to surmise that the phenomenon of adaptation of the flash response to increased pressure might enable a luminescent ctenophore to descend gradually from the surface of the sea to such depths without having the intensity of its flash reduced by the increase in pressure.

The similarities between the flash of *Mnemiopsis* and that of *Chaetopterus,* as well as similarities in the effects of pressure on the two, are more impressive than the differences, which are largely quantitative. The flash of the latter organism is of the order of 100 times longer in duration, but in both organisms the decay after peak intensity is exponential, and in both organisms the time-course becomes longer with decrease in temperature. The *Chaetopterus* flash, as well as luminescence intensity of the secreted slime, are somewhat less sensitive to increased pressure than is the *Mnemiopsis* flash, while the effects of increased pressure are more sensitive to temperature and to the presence of urethane.

The foregoing remarks are necessarily somewhat general. Further and more specific interpretation of the observed phenomena must await more detailed knowledge than is presently available concerning the total process of the flash response. The effects of pressure on the luminescence of homogenates ("squeezates") of *Mnemiopsis* have been studied and will be made the subject of a later communication.

SUMMARY

Using small segments of excised meridional canals of *Mnemiopsis leidyi,* luminescent flashes induced by square wave electrical pulses of precise voltage and duration have been accurately recorded with the aid of a photomultiplier-amplifier and dual beam cathode ray oscillograph. Analyses of more than 8000 flashes, under various conditions of temperature, hydrostatic pressure, urethane concentration, and frequency of repetitive stimulation, have led to the following generalizations.

1. The time course of luminescence intensity in an individual flash at a given temperature is not appreciably altered by increased pressures which greatly reduce the flash maximum.

2. The latent period between time of stimulation and onset of response is likewise not significantly altered by pressure, within the sensitivity of the methods employed.

3. In a series of consecutive flashes, at frequencies of one per 3 seconds to 4 per second, the initial effect of increased pressure is always to reduce the maximum intensity of the flash; detectable reductions are caused by relatively slight pressures, of less than 100 psi.

4. A series of pressure increases in increments of several hundred psi is less inhibitory on flash intensities than a sudden increase to the highest pressure involved. Sudden increases of 1000 to 5000 psi temporarily abolish the flash, whereas with gradual increases to these pressures, the flash may persist, though at reduced intensity.

5. Under a sustained pressure, a process of adaptation frequently occurs, whereby on continued repetitive stimulation the initially inhibited flash recovers and then facilitates, sometimes to much higher intensities than prior to compression.

6. On sudden decompression, part way or all the way to atmospheric pressure, the initial effect is always an increase in flash intensity over that occurring under pressure, or sometimes over that occurring prior to compression. The only exception occurs when excitability has disappeared completely, as occasionally happens.

7. The recovery process after pressure assumes a variety of unpredictable patterns; in some instances the first one to three flashes are excessively high ("overshoot"), followed by rapidly decreasing flash maxima, whereas in other instances a gradual facilitation and decline take place.

8. Excess luminescence intensity in the recovery phase is independent of maintaining repetitive stimulation during the preceding period under pressure.

9. Excitability that has been lost through fatigue or unknown causes can be restored in some instances by subjecting the specimen to pressures of 3000 to 5000 psi for periods of 1 to 5 minutes.

10. Qualitatively the same results of pressure are observed at various temperatures between 15 and 35° C. Any definite influence of temperature on the effects of pressure is obscured by variations in the quantitative effect of a given pressure on different specimens and on the same specimen in different physiological states.

11. Urethane, in concentrations between 0.05 and 0.5 M, causes a progressively increasing reduction of flash maxima with duration of exposure of the drug, and at rates that increase with drug concentration and temperature. Qualitatively the same phenomena are observed with respect to the influence of pressure on the flash in the presence as in the absence of urethane.

12. The *Mnemiopsis* photogenic system is particularly sensitive to destructive effects of urethane and of elevated temperatures, thereby obscuring the possible existence of reversible thermal inactivation reactions and the possible influence of pressure thereon.

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OSMOTIC HATCHING IN THE EGGS OF SOME FRESH-WATER COPEPODS

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Hatching of the eggs of eucopepods apparently has been described only by Marshall and Orr (1954, 1955) and Ziegelmayer (1927). The two papers differ fundamentally in the interpretation that is given, for Ziegelmayer, working with 17 species of *Cyclops,* thought the outer egg membrane swelled over a period of 6 to 12 or more hours, developing a pressure between the inner and the outer membranes. Subsequently, according to him, the outer membrane would burst, and the nauplius emerge, closely surrounded by the inner membrane. On the other hand, Marshall and Orr thought that the inner membrane swelled, and that the pressure developing within it resulted in the rupture of the outer membrane. The inner membrane, containing the unhatched nauplius, emerged through the opening. These authors described a considerable space between the nauplius and the stretched inner membrane. Observations were made by Marshall and Orr (1954) primarily on the marine calanoid, *Calanus finmarchicus,* but they supplemented their study by the examination of other marine copepods belonging to four sub-orders (Calanoida, Cyclopoida, Harpacticoida, and Caligoida), and by the examination of two species of fresh-water cyclopoids (*Cyclops agilis* and *C. viridis*).[1]

From the general appearance of the hatching process, both Ziegelmayer and Marshall and Orr concluded that it was osmotically controlled. Although Ziegelmayer's paper was devoted largely to reports of experiments on the permeability and changes of permeability of the membrane, he performed no experiments that were aimed at proof of osmotic control. Marshall and Orr performed a rather inconclusive experiment, in which they placed 15 *Calanus finmarchicus* eggs that were nearly ready to hatch in sea water that had been diluted with a small quantity of fresh water. Eleven of the eggs bulged and 7 hatched successfully, whereas in the controls in undiluted sea-water only 3 out of 14 bulged and hatched.

In explanation of the onset of the hatching process, Ziegelmayer was convinced that there was a change of the permeability of the membrane, caused by some influence (hormone?) from within (hence, from the enclosed nauplius). On the other hand, Marshall and Orr (1954) suggested that it (p. 400) "might be that a sudden increase of excretion by the embryo leads to an increased content of salts and the imbibition of water."

MATERIALS AND METHODS

Ovigerous specimens of *Diaptomus siciloides, D. ashlandi, D. oregonensis, Cyclops bicuspidatus* and *Mesocyclops edax* were taken from the plankton in

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[1] After the present paper was in press, it was discovered that P. Heegaard in 1947 (Contribution to the phylogeny of the arthropods: Copepoda, in *Spolia Zool. Mus. Hauniensis,* **8**: 1–227) had given figures and brief descriptions clearly indicating that hatching in *Caligus curtus, C. rapax,* and *Lernaeocera branchialis* occurs in a manner comparable to that described by Marshall and Orr (1954).

Hatchery Bay, Put-in-Bay, Ohio (western Lake Erie) in mid-June to mid-July, 1958 (air and water temperatures were 21° ± 1° C. during the period of collection and observation – 23° C. for *M. edax*). Most of the observations, and all of the experiments, were with *Diaptomus ashlandi* and *D. siciloides*. Specimens were kept in U. S. Bureau of Plant Industry model watch-glasses until chosen for detailed observations, at which time the egg sacs were removed from the mothers and the eggs observed with a compound microscope magnifying 100× and 443×. No coverslip was used, the objective being immersed directly into the water when necessary.

Observations of the hatching procedure were supplemented by experiments designed to test the validity of the osmotic theory of hatching. Sucrose solutions were made up of the following concentrations: 1 *M*, 0.5 *M*, 0.4 *M*, 0.3 *M*, 0.2 *M*, 0.1 *M*, 0.05 *M*, 0.04 *M*, 0.03 *M*, 0.02 *M*, and 0.01 *M*. These solutions were used to ascertain the approximate osmotic value of the fluid within the inner membrane, and to test the permeability to water of the inner membrane and larval surface at certain stages of the development of the nauplius in relation to the moment of hatching. To avoid repetition, the detailed experimental procedures are more conveniently given below under Results.

The work for this paper was undertaken at the F. T. Stone Laboratory, and was supported by a research stipend furnished by the Ohio Division of Wildlife, through the Ohio Natural Resources Institute, Charles A. Dambach, Director. Sincere thanks are extended to Dr. Dambach, and to Dr. Loren Putnam, Director of the Stone Laboratory, for their unstinted aid in providing space and equipment for the accomplishment of the project.

Results

1. *Simple observations*

No difference was observed in the hatching of the eggs of *Diaptomus siciloides*, *D. oregonensis*, and *D. ashlandi*. Before hatching, the eggs averaged 109 μ in diameter, and thus had a volume of 678,110 μ^3. Individual eggs were separated from each other by the material of the egg sac proper, as shown in Figure 1, which depicts a portion of an egg mass of *D. siciloides*, with the eggs near the hatching stage. In this preparation the eggs were spread apart with fine needles for ease in viewing them, and one has been displaced from the egg sac. As shown, there are spaces of small but variable magnitude between the egg sac material and the eggs proper. Only a single membrane can be distinguished around the enclosed larva but in reality there are two, as shown below. The contained embryo for the most part fills the entire space within the membranes, although when viewed from the dorsal aspect, the naupliar appendages are visible laterally, closely appressed against the body. The red bi-crescentic naupliar eye is clearly visible anteriorly. The nauplius was observed to twitch its legs from time to time as much as 24 hours before hatching began.

The initiation of hatching was indicated by the appearance of a fluid-filled space between the nauplius and the egg membranes. This was followed very quickly by the bulging of the egg surface (Fig. 2). The outer membrane broke, probably due to the internal pressure, and it could be seen that there was a second inner membrane protruding through the opening (Fig. 3). In eggs which were isolated from the egg mass, it was clear that the two halves of the outer membrane were pushed aside by the emerging inner membrane. For some time a portion of the inner membrane remained inside of one of the halves of the outer membrane (Fig. 4), but eventually the entire inner membrane slipped out as a perfect sphere, and left the outer membrane behind (Fig. 5). For all of this period the volume enclosed by the inner membrane was increasing, so that when the stretched membrane slipped free of the outer membrane it had an average diameter of 153 μ and a volume of 1,875,400 μ^3. Thus the volume, compared to the original volume of the egg, increased in a ratio of 2.77:1. The nauplius was completely surrounded by a fluid-filled space.

During the extrusion of the inner membrane from the outer, the unhatched nauplius typically remained completely motionless. In some instances it twitched, but never more than it had for several hours previously. At first after extrusion, the three pairs of appendages were held as they were before the swelling of the inner membrane. Although each larva that was observed at this stage was examined carefully, and larvae were seen from all possible angles, no trace of a third membrane, close around the animal, could be detected. After a period of one or two minutes, the appendages suddenly broke free from the sides of the body, and became extended laterally in their usual free-living naupliar position. This was observed many times. When the larva was viewed from a dorsal or ventral position, it could be seen that the appendages broke free from the sides of the body in a series of two or three short jerks. There was no evidence that this movement took place through muscular contractions of the animal (though this was not precluded). The *appearance* was that the appendages were, due to their structure and elasticity, pulling in the direction of their normal naupliar position, and that suddenly some tissue, membrane, or other material holding the appendages down tore loose from

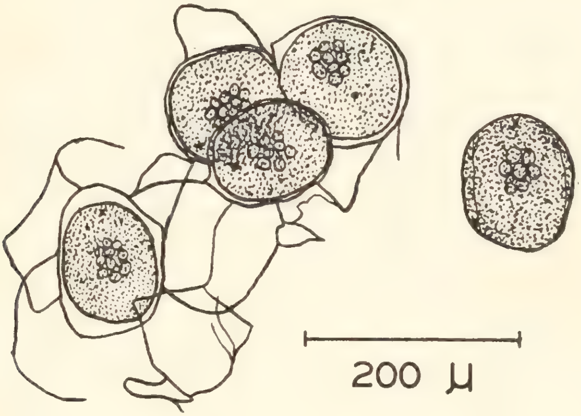
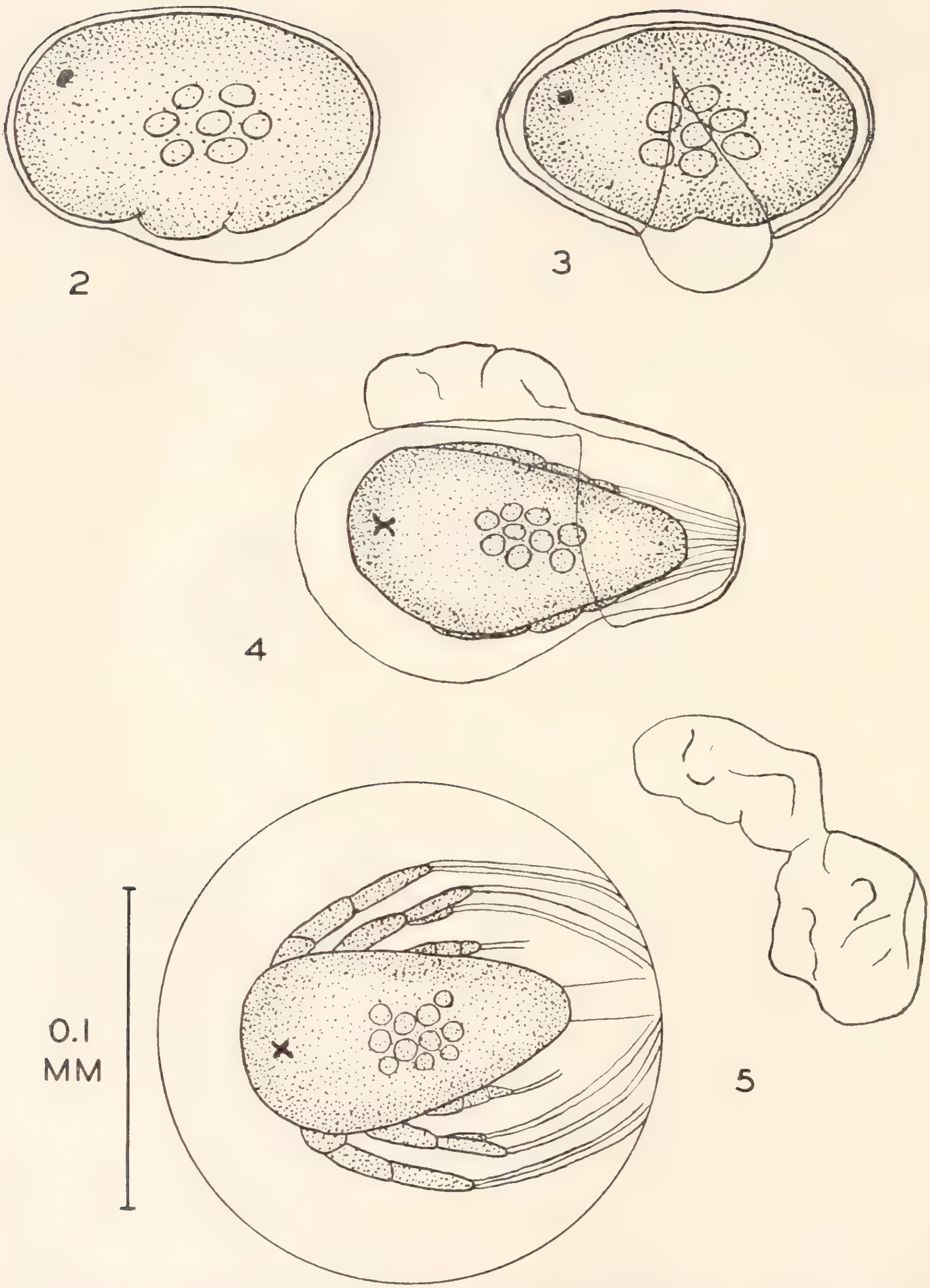


FIGURE 1. A portion of an egg sac of *Diaptomus siciloides* with eggs near the hatching stage. The sac was teased apart with fine needles, and one egg has been displaced from it. Note that the individual eggs lie somewhat loosely within the secretion forming the egg sac. Also note that the embryo is closely invested by the egg membranes, so that there is no space between the membranes and the enclosed nauplius.



FIGURES 2–5. Individual eggs of *Diaptomus siciloides* during hatching. Figure 2: a fluid-filled space has appeared between the egg membranes and the nauplius, and the membranes are bulging on one side. The inner and outer egg membranes are not yet distinguishable. Figure 3:

the strain. A few seconds after the appendages assumed their naupliar position, the animal began to move them in the twitching manner characteristic of free-swimming calanoid nauplii. Approximately a minute later the diaphanous membrane of the sphere burst with great suddenness. The internal fluid, being under considerable pressure, was forced almost explosively out through the breach, carrying the nauplius with it. While the nauplius was within the sphere, the setae of the appendages, and the setae at the posterior end of the animal, appeared as though they could easily and readily perforate the delicate membrane, for they impinged upon its surface as the animal continued its movements. However, this method of escape apparently did not occur during normal hatching, for almost invariably the larva escaped head first, whereas the setae touched the membrane at the opposite side of the sphere. Upon close observation it could be seen that the end of each of the setae was pliable and bent over when it touched the membrane. Penetration by the sharp ends of the setae as suggested by superficial examination, could not occur (see below in section 4 for further observations on the breaking of the membrane).

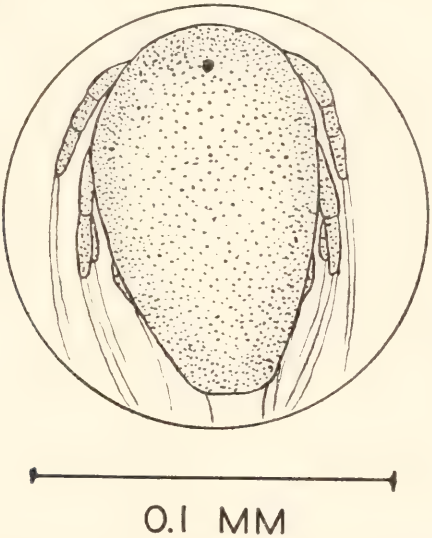


FIGURE 6. The nauplius of *Cyclops bicuspidatus* within the sphere formed by the inner egg membrane after extrusion.

The total time elapsing from the first indication of hatching to its completion ordinarily varied from 7 to 8 minutes.

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the outer egg membrane has burst, and the inner membrane is bulging out through the opening. The outer and inner membranes are clearly seen. Figure 4: the outer membrane covering the anterior half of the emerging larva has slipped off, but the inner membrane remains within the other half of the outer membrane. The naupliar appendages are closely appressed against the sides of the body. Figure 5: the inner membrane, expanded to its maximum diameter, has slipped out of the outer membrane entirely, forming a perfect sphere. The naupliar appendages have assumed their swimming position.

Hatching was observed in single sets of eggs from *Cyclops bicuspidatus* and *Mesocyclops edax*. It occurred in much the same manner as in the 3 species of *Diaptomus,* and took an average of 6½ minutes from start to finish. As with *Diaptomus,* the nauplius was passive during the swelling and protrusion of the inner membrane from the outer membrane and from the egg mass, except for a few twitches. The volume increase of the contents of the inner membrane was not quite as extensive as in *Diaptomus*. Before hatching the eggs averaged 82 μ in diameter. The spheres averaged 112 μ. Hence the average volume changed from 288,710 μ^3 to 735,655 μ^3, or a ratio of 1:2.55. The nauplius occupied a greater portion of the contents of the sphere than was the case with *Diaptomus* (Fig. 6), and it did not escape in the same explosive fashion. When the membrane surrounding the sphere burst, there was a sudden release of pressure, and the sphere collapsed, opening, as with *Diaptomus,* at the head end of the larva. In both of these cyclopoids, in all the instances observed, however, the nauplius remained somewhat entangled in the membrane, and escaped only after a short struggle.

2. *Experiments indicating the osmotic nature of hatching*

The general appearance of the hatching process in copepods strongly suggests that it is osmotically regulated. However, this has not been proven incontrovertibly by any experimental results heretofore reported.

In preliminary exploratory experiments, eggs of *Diaptomus siciloides* in the beginning stages of hatching were immersed in a 1 *M* solution of sucrose, or in double-distilled water. The permeability to water of the inner membrane and the naupliar surface was clearly shown by the fact that in the sucrose solution the larvae and the inner membranes shrank drastically from the outer membrane through the osmotic loss of water to the hypertonic solution. Obviously the larvae were destroyed (Fig. 7). In double-distilled water, on the other hand, hatching (*D. siciloides*) was completely normal except that the average time consumed during the hatching process was reduced to 6 minutes, compared to an average of 7½ minutes for the controls (the nauplii from the experimental eggs became turgid and weak in their movements after hatching, and died by bursting in 15 to 20 minutes).

A 0.1 *M* sucrose solution was used for another set of eggs. Some of the eggs already had hatched, one was in the process of hatching, and a group of five in the egg mass had not yet begun to hatch. The inner membrane of the hatching egg quickly shrank back against the larva. None of the larvae was obviously distorted from the osmotic effects of the solution, and they continued to twitch in a normal fashion. No sign of hatching was observed in any of the eggs. The eggs were maintained in the 0.1 *M* sucrose for 4 hours, at which time they were transferred to lake water. Immediately all of them began to swell. Spheres of normal size formed, but they were not entirely freed from the outer membranes. The nauplii were very weak. All of them hatched, but they died soon. It is thought that these deaths may have been the result of some other factor than osmotic effects, for example from anoxia. This is suggested by subsequent experiments and by the fact that some of the already hatched siblings of the experimental nauplii were placed in 0.1 *M* sucrose for 24 hours, then transferred to lake water, with no ill effects.

A set of eggs of *D. siciloides*, some of which were hatching, was placed in 0.05 *M* sucrose solution. Some larvae began the hatching process. There was some swelling, but apparently insufficient pressure was built up to burst the outer membrane. The eggs were placed back in lake water after 30 minutes in the sugar solution. Hatching began immediately, the first larva being freed 8 minutes later.

Another set of eggs of the same species at hatching time was placed in 0.04 *M* sucrose solution. Those that had already formed spheres hatched. Those still in the outer membranes (including those that had started to hatch) failed to hatch or change in any way during 17 minutes. The eggs were then placed in 0.03 *M* sucrose. Swelling was immediate (in 3 out of 4 eggs). One of these hatched in about three minutes. Another swelled considerably but failed to squeeze out of the outer membrane. There was no further change for 15 minutes. The remaining eggs were then placed in 0.02 *M* sucrose. In 2½ minutes one larva had hatched, but when the membrane broke, the larva was not thrown out. In another egg a sphere was formed (136 μ in diameter—considerably smaller than the average). When the membrane broke, it did so slowly, taking a full two seconds to collapse. The larva was not thrown out in the usual manner, but temporarily remained entangled in the collapsed membrane.

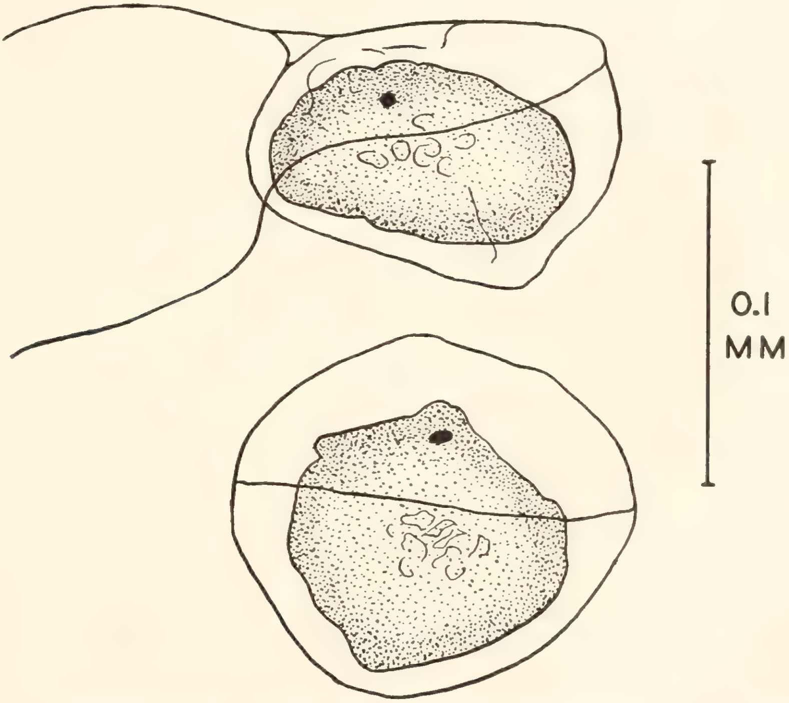

FIGURE 7. Two hatching eggs of *Diaptomus siciloides* after immersion in 1 *M* sucrose solution. The nauplii and the inner membranes have collapsed through the osmotic loss of water. The outer membranes are unshrunken, though somewhat contorted.

The results described above are summarized, along with additional information, in Table I.

The resistance of the outer membrane of eggs in the early stages of hatching interfered with efforts to ascertain the approximate osmotic pressure of the fluid within the expanded inner membrane. It was necessary to experiment with spheres that already had been extruded (thus very shortly before completion of hatching). Successful observations were completed in 18 instances, results being similar both for *D. ashlandi* and *D. siciloides*.

The results obtained with isolated extruded spheres are condensed in Table I. The following are representative experiments: 1) A nauplius of *D. ashlandi* in the twitching stage in a sphere was placed in 0.05 *M* sucrose. The diameter of the sphere decreased very gradually over a period of 12 seconds (plus an unknown portion of the duration of time needed to find it under the microscope). When all the space within the membrane had disappeared, the larva moved, puncturing the membrane with one of its antennae, after which it escaped. 2) A nauplius of *D. ashlandi* in a sphere was placed in 0.04 *M* sucrose. The sphere shrank. It was then placed back in lake water where it swelled up again. It was placed in 0.04 *M* sucrose a second time, and shrank again. The nauplius then moved, punctured the membrane, and escaped. 3) A larva of *D. ashlandi* in a sphere was placed in 0.03 *M* sucrose. The membrane shrank somewhat, but a considerable space remained between the nauplius and the membrane. The membrane no longer formed a perfect sphere, but was distorted to an ovoid shape with dimensions of 119 μ × 146 μ. The larva hatched successfully.

Table I

Summary of the effect of various concentrations of sucrose on the swelling and hatching of eggs. (+ = occurring, ± = sometimes occurring, and sometimes not, − = not occurring.)

Sucrose conc. (*M*)	Swelling of intact egg	Bursting of outer membrane	Extrusion of inner membrane	Swelling of inner membrane	Hatching from extruded sphere	Shrinking of inner membrane
Lake water	+	+	+	+	+	−
0.01	+	+	±	+	+	−
0.02	+	±	±	+	+	−
0.03	+	±	±	±	±	±
0.04	−	−	−	−	±	+
0.05	−	−	−	−	±	+
0.10	−	−	−	−	−	+

From the results of such experiments, it appears that the osmotic pressure of the fluid within the extruded inner membrane was approximately equivalent to that of 0.03 *M* to 0.04 *M* sucrose. Such solutions have osmotic pressures of 0.672 to 0.896 atmosphere. The Δ of the fluid (Δ_f) would be 0.056 to 0.074. The freezing point of the Lake Erie water used was −0.03° C. The Δ of the internal fluids of nauplii has never been measured, but Δ_i for fresh-water Crustacea, as summarized by Krogh (1939) and Harnisch (1951), lies between 0.30 (*Daphnia magna*) and 0.81 (*Potamobius*). Przylecki (1921), reported by Krogh (1939), observed the Δ of older eggs (50–80 hours) of *Daphnia magna* to be 0.74. It would therefore appear that Δ_i is greater than Δ_f, which in turn is greater than Δ_o.

The above reported results indicate very clearly that the hatching of the copepod eggs studied here was by osmotic means.

3. *Test of the permeability of the egg membranes*

Ziegelmayer (1927) concluded that a change in the permeability of the (outer) membrane initiated the process of hatching. In contrast, Marshall and Orr (1954) suggested the possibility that there was a sudden increase of the osmotic pressure of the fluid within the inner membrane, after which hatching proceeded. To the present author this latter hypothesis seemed reasonable, for the alternate hypothesis apparently would be that the non-living inner membrane would have to change its permeability suddenly. Such a sudden change certainly would not be unexpected in a living membrane, but would not be as likely for a non-living membrane. Therefore the results reported below were unexpected.

As a preliminary experiment, to test the effect of some of the higher osmotic concentrations on the nauplius itself, larvae of *Diaptomus siciloides* which had just hatched were placed in a series of sucrose solutions as follows: 1 *M*, 0.5 *M*, 0.4 *M*, 0.3 *M*, 0.2 *M*, and 0.1 *M*, with other nauplii remaining in lake water as controls. Larvae became contorted and succumbed instantly in 1 *M* sucrose. In a 0.5 *M* solution they died within a few seconds, and likewise showed distinct evidence of the osmotic removal of water from their tissues. Results were the same in 0.4 *M* sucrose, though the larvae lived somewhat longer. In 0.3 *M* sucrose they lived over ten minutes, but the end result was similar. In the 0.2 *M* solution, at the end of 10 minutes they appeared normal, but moved seldom and weakly. Subsequently they died. In the 0.1 *M* solution they lived normally for many hours, but moved somewhat less vigorously than did the controls.

Thus the larvae can withstand a solution with an osmotic pressure as great as that of 0.1 *M* sucrose, but not as great as that of a 0.2 *M* solution.

In a subsequent experiment, an egg sac of *D. ashlandi* containing hatching eggs was placed consecutively in 0.1 *M*, 0.2 *M*, 0.3 *M*, 0.4 *M*, and 0.5 *M* sucrose, and observed for shrinkage of the inner membranes and of the enclosed nauplii. No shrinkage was apparent in the 0.1 *M* or 0.2 *M* solutions. A slight shrinkage could be seen in the 0.3 *M* solution, but it was somewhat obscure. In 0.4 *M* and 0.5 *M* solutions, however, shrinkage was considerable. The nauplii within the shrunken inner membranes appeared to be destroyed.

However, in three out of the ten eggs in the egg case, no shrinkage occurred, even in the 0.5 *M* solution, and the enclosed nauplii continued to twitch. A few minutes after being transferred to 0.5 *M* sucrose, one of the three suddenly began to shrink (not timed) but the other two remained as they were. Approximately one-half hour later the second one rather suddenly shrank. The third, on the other hand, still maintained life, and was intact, at the end of 2½ hours, although by this time (in the conditions of the experiment) considerable evaporation had occurred, and therefore the sugar concentration was higher than 0.5 *M*.

A second egg case of *D. siciloides* was placed in 0.5 *M* sucrose. Again, three out of ten eggs failed to shrink in the 0.5 *M* solution, but the remainder clearly showed the effects of the hypertonic external medium. Two of the three started to shrink 19 minutes after the egg case was placed in the sucrose, and the other began 2 minutes later. The process of shrinking took approximately 3 to 4 minutes (the time at which shrinking was completed was difficult to judge exactly). One-half hour after the egg case was placed in the sucrose, it was returned to lake water. Hatching was successful in nine of the ten eggs, though the nauplii were not normal (see below in section 4 for a more detailed description of this hatching).

These results suggest that in those eggs where hatching had begun, or was ready to begin, the inner membrane rather suddenly became permeable to water, whereas in those eggs not yet ready to hatch the inner membrane was impermeable.

To test the hypothesis that a permeability change takes place when the nauplius is ready to hatch, some eggs definitely not yet to the hatching point were tested by placing the egg cases in 0.5 *M* sucrose solution. The eggs of *Diaptomus ashlandi*, laid less than two hours previously by a gravid female, failed to shrink although they remained in the solution for an hour.

Two egg cases from *D. siciloides* were tested. Both contained eggs with embryos that were twitching and with eyes that were fully developed. In neither did shrinking take place at first. In one egg case there still was no shrinking after 17¼ hours, at which time it was replaced in lake water. No hatching took place, though the embryos were alive, as shown by the fact that they continued to twitch. After 8 hours the eggs were again placed in sucrose. Again no shrinkage occurred. When removed to lake water 16 hours later, the eggs appeared normal except that there was no twitching, but before long the embryos disintegrated.

In the second egg case containing twitching nauplii there was no shrinkage in 0.5 *M* sucrose at the end of an hour. In 2½ hours, however, 3 of the 16 eggs were shrunken. This egg case was thereupon return to lake water. By the time the solution was changed and the eggs located under the microscope, the shrunken eggs had swollen again, and one was beginning the process of hatching. Hatching then continued in egg after egg, and was perfectly normal in all instances except one, where the inner membrane after extrusion must have been perforated only slightly and lost its internal pressure slowly, collapsing completely around the larva, which struggled for a few seconds before it broke out.

These observations confirm that there is a change of permeability of the inner egg membrane at the time of hatching.

4. *Observations on the bursting of the inner egg membrane*

As discussed above, the nauplius always began its characteristic movements in normal hatching about a minute before the inner egg membrane burst and liberated it. It appeared as though the nauplius ruptured the membrane in some way by its activities, although it was not clear how this was done inasmuch as the rupture almost always occurred at the head end of the nauplius. Marshall and Orr (1954) said of this final act of hatching: "Quite suddenly it [nauplius] tears the membrane and swims away" (p. 393). Similarly, Ziegelmayer (1927) stated that the larva ruptured the inner membrane by the movements of its second antenna, but he thought the inner membrane was closely appressed around the nauplius after it was liberated from the outer membrane passively by an explosion-like bursting of the latter. In the observational section (section 1 above) of the present paper, the rupture of the membrane was implied to be the result of the struggling of the nauplius, because this was the way it appeared.

However, one of the experiments unexpectedly gave very revealing results. As reported in section 3 (above), an egg case in which the eggs were in the process of hatching was placed in 0.5 *M* sucrose for half an hour, then replaced in lake water. All the eggs but one hatched, but the nauplii were very weak. As many of the hatchings as possible were watched carefully and continuously until hatching was completed. If the nauplii twitched or moved at all during hatching, they did so only by very slight and slow movements of the appendages. Three hatchings were followed where the entire process took place with no evidence of any muscular movement whatsoever on the part of the nauplius. Two other cases were similar, but there were some slight movements. These, however, were by no means sufficient to burst the membrane. The remaining hatchings could not be followed throughout (and one egg did not hatch). In spite of lack of naupliar movements, at the proper time the inner egg membrane burst and the nauplii were liberated.

A second egg case (*D. siciloides*) in which hatching was taking place was treated in the same manner. Of the 9 eggs in the egg sac, 8 shrank at once or very soon after immersion in the sucrose solution. One however, shrank only just before the case was returned to lake water, one-half hour later. In the lake water all of the 9 eggs hatched, although three of them had been "hatched" artificially by the inevitable rough treatment of rapidly changing solutions (these three, although appearing normal, never moved after liberation). Of the remaining six eggs, four hatched without any movements, and after hatching, three of these never moved (the fourth moved its appendages slightly during the process of dying, immediately after hatching). One of the nauplii twitched regularly, though weakly, before hatching, but during the period of the final bursting of the membrane there was no further movement, and the larva never moved after hatching. Only one of the nauplii (presumably from the egg that shrank at the last minute) hatched normally and lived indefinitely after hatching.

It is not believed that the bursting took place through the continued swelling of the sphere. Both before and after the above observations were made, numerous attempts were undertaken by measuring extruded spheres in normal eggs, to ascertain whether the swelling of the sphere continued until the time of breaking. No evidence of such growth after extrusion was obtained.

5. *Attempts to demonstrate the existence of a hatching enzyme*

In the observations and experiments described above, the hatching eggs were immersed in less than 0.5 cc. of lake water during hatching. There never was any evidence that the liberation of a hatching enzyme by the bursting of hatching eggs speeded up the hatching of those eggs in the cluster that still remained unhatched. In the eggs of *Diaptomus,* as reported above, the volume of the fluid within the inner egg membrane just before the nauplius was freed averaged 1,875,400 μ^3. This is less than 1/300,000 the volume of 0.5 cc. ($= 5 \times 10^{11}\ \mu^3$). With such a dilution of any hatching enzyme that might be present, one would hardly expect an effect.

Therefore, the volume of water involved was reduced (three experiments on *D. siciloides*) by drawing detached egg sacs in which the eggs were actively hatching into capillary tubes (i.d. = 1 mm.), along with half of an egg sac in which no hatching was occurring. The other half of the non-hatching batch of eggs was kept as a control.

In one of the three experiments the experimental eggs were in a rather early developmental stage. There were 10 experimental eggs and 15 hatching eggs enclosed in the capillary tube, with $12.7 \times 10^9\ \mu^3$ of water. Hence the ratio of fluid from the bursting membranes to the amount of diluting water was approximately 1:450. Neither the experimental eggs nor the controls hatched.

In the other two similar experiments the experimental eggs were in a very late stage of development. During the experimental period, hatching occurred in the experimental eggs some time after the other eggs had hatched. However, hatching took place almost simultaneously in the controls (in both instances hatching began first, and was completed first, in the experimental eggs, but the difference is not thought to be significant, inasmuch as some of the control eggs hatched before the last of the experimental ones). Thus, the existence of a hatching enzyme was not clearly demonstrated, in the conditions of these experiments.

Discussion

The results reported in the present paper fully confirm the osmotic nature of the hatching process in the eggs of copepods. The observations of Marshall and Orr (1954) on the events of hatching are supported and supplemented. No evidence was obtained in support of Ziegelmayer's (1927) contention that the outer membrane expanded osmotically while the inner membrane remained closely appressed around the enclosed nauplius. Furthermore, the observation reported by Ziegelmayer that the membrane began swelling 6 to 12 hours before hatching was not confirmed. Repeated attempts to detect an increase in volume of the egg previous to the few minutes before the hatching process was completed gave negative results. Marshall and Orr (1954) stated concerning the discrepancy between their observations and those of Ziegelmayer (p. 400): "It is difficult to decide whether Ziegelmayer was unable to see the bulging out of the inner membrane or whether the specimens he examined behaved in a different way." Ziegelmayer studied 17 (unlisted) species of *Cyclops*. Inasmuch as Marshall and Orr observed hatching in *Cyclops agilis* and *C. viridis*, and I observed it in *C. bicuspidatus* and *Mesocyclops edax*, and the behavior of all these was unlike that reported by Ziegelmayer, it would appear that his observations were faulty or deficient, either through the use of too little magnification, or through failure to follow through hatching in individual eggs.

On the other hand, the results reported above support Ziegelmayer's belief that hatching is initiated by a change of the permeability of the membrane. The lack of a similar conclusion by Marshall and Orr undoubtedly is associated with their lack of extensive experimentation.

From the above, two unsolved questions arise: 1) what is the origin and the nature of the dissolved material within the inner egg membrane that gives rise to a Δ_f of this fluid greater than the Δ_o of the external medium, and 2) what is the cause of the sudden change in the permeability of the inner membrane?

In fresh-water copepods, such as those reported here, the osmotic pressure of the fluid within the inner membrane conceivably could have its origin simply in the attainment of an equilibrium between Δ_f and Δ_i. However, the hatching of marine copepods, as reported by Marshall and Orr (1954), occurs in the same manner as that of the fresh-water forms. In most marine invertebrates the osmotic pressure of the internal medium is in equilibium with that of sea water, and there is no reason to believe that such species as *Calanus finmarchicus*, *Metridia longa*, and *Euchaeta norvegica*, which are stenohaline, are an exception. Therefore, in marine species, Δ_f must be greater than Δ_o, and greater than Δ_i at the time the egg was laid. No information exists at present bearing on the relation of Δ_i of the nauplius to Δ_f in these marine species just before hatching. Δ_i might either equal Δ_f., or it might be less than Δ_f. A Δ_f that is greater than Δ_o can be attained only by the action of the embryo or larva enclosed in the egg. It could result, as suggested by Marshall and Orr (1954), through the excretion of metabolic wastes by the embryo and/or nauplius, or there could be an active secretion of substances with osmotic value by special glands or gland cells (or both of these processes could be involved simultaneously). In view of the sudden change of permeability of the inner egg membrane described above, if excretory products are involved they need not be excreted suddenly as postulated by Marshall and Orr, but could accumulate gradually, and become osmotically effective suddenly through the rapid alteration of membrane permeability that initiates hatching. These matters can be settled only through further experimentation, particularly on stenohaline marine species of copepods.

It appears unlikely that a non-living membrane, such as the inner egg membrane of copepods, would be so constituted that its chemical or colloidal nature would suddenly be altered spontaneously at the proper time for hatching. If there is no spontaneous change, the influence for the alteration must come either from outside, or from the larva inside. Conceivably, the chemical nature of the membrane could be such that bacterial action from without would alter it in a definite course of time, but such an adaptation in evolution (especially considering that the bacterial population of waters is far from constant) seems far less likely than the evolution of a special hatching enzyme whose function is the chemical alteration of the membrane. Such a chemical alteration could change the membrane into a semi-permeable membrane (permeable to water) from its initial impermeable condition. It is true that the preliminary experiments described in section 5 above failed to detect the presence of such a hatching enzyme, but these experiments need repetition and refinement, and furthermore, from the nature of the experiment, negative results are not conclusive (although positive results would have been). The presence or absence of glands or gland cells producing a hatching enzyme has not been ascertained, but should be demonstrable histologically.

Hatching enzymes, such as are postulated above for the hatching of copepod eggs, have been proven to exist in certain fish eggs. Here, however, hatching apparently does not involve osmotic phenomena. The hatching enzyme, which is produced by special embryonic glands, digests the egg membranes, and the fry emerges more or less without the benefit of its own muscular movements (*e.g.*, see Bourdin, 1926 and Privolnev, 1943). Similar enzymes have been proven to exist in eggs of other aquatic animals, including *Rana pipiens* (Cooper, 1936). On the other hand, Wilson (1958) obtained results very similar to my own in the hatching of the eggs of the nematode, *Trichostrongylus retortaeformis*. Despite his negative results, he concluded from qualitative observations that some "hatching factor" is secreted which weakens the protein membrane before hatching. The hatching process itself in *T. retortaeformis* he thought to be osmotically determined.

Both Ziegelmayer and Marshall and Orr described the final rupture of the inner membrane as due to the active movements of the nauplius. The present results contradict this, and show that the hatching process can proceed to completion without any movements on the part of the enclosed nauplius. Although there apparently was no further increase of the volume of the fluid enclosed by the inner membrane during the final period of the hatching act, the possibility is not eliminated that there was a continuation of the entry of osmotic water. With the membrane already stretched to its physical capacity, such a further entry would build up the internal pressure to the bursting point of the membrane. A further hypothesis suggests itself, however, namely that the membrane is destroyed chemically by a secretion from the anterior end of the larva. This would account for the fact the membrane almost invariably burst at the head end of the nauplius. These hypotheses also can be tested only by further experimentation.

Pyatakov (1926) studied hatching in the arguloid, *Argulus foliaceus*. Although his paper dealt primarily with the formation of the seam in one of the egg membranes along which splitting occurred during hatching, it is clearly implied that the hatching process itself is similar to that occurring in the Eucopepoda. Ziegelmayer (1927) reported, but did not describe, osmotic hatching in the eggs of an isopod (*Asellus*) and in an anostracan (*Branchipus*). Hall (1953) described hatching in the anostran *Chirocephalus,* and suggested that osmotic factors were involved. Przylecki (1921) and Ramult (1925) have presented results, summarized by Krogh (1939) and by Needham (1931), showing that in certain Cladocera, hatching is by osmotic means. In these forms, however, hatching differs considerably from that of the Copepoda, for it is the embryo itself that swells osmotically, and its increase in volume stretches the egg membrane until it bursts. A similar method of hatching was reported by Manton (1928) for *Hemimysis lamornae*. In some unpublished observations, the present author determined that hatching in the fresh-water decapod, *Palaemonetes kadiakensis*, occurs in part through osmosis. For a discussion of and references concerning osmotic hatching in other invertebrates see Needham (1931).

On the other hand, all Crustacea do not hatch osmotically. Le Roux (1933) described hatching in the amphipod *Gammarus,* where the young emerges from the egg by the active use of special egg teeth on the telson. This method was corroborated by the present author in the examination of hatching in *Gammarus fasciatus* in western Lake Erie.

SUMMARY

1. The hatching process is described for the fresh-water copepods *Diaptomus ashlandi, D. siciloides, D. oregonensis, Cyclops bicuspidatus*, and *Mesocyclops edax*. In all of these species the inner membrane expands by the osmotic entry of water. The internal pressure thus produced ruptures the outer membrane, and the inner membrane containing the nauplius is extruded, forming a sphere whose volume is more than 2½ times that of the original egg. Subsequently the inner membrane bursts and the nauplius is thrown out.

2. It is shown that the osmotic pressure of the fluid within the expanded inner membrane is equivalent to that of a 0.03 to 0.04 *M* sucrose solution.

3. The inner membrane remains impermeable to water until the egg is ready to hatch. Thereupon the membrane changes its permeability within a short period of time. Hatching can be prevented indefinitely in eggs that are ready to hatch by immersing them in sufficiently concentrated sucrose solution.

4. Although during normal hatching the nauplius is active for a period of approximately a minute before hatching, this activity is not necessary for the completion of the hatching act. Nauplii hatched, even though they had been completely immobilized.

5. Attempts to demonstrate the presence of a hatching enzyme were unsuccessful.
6. It is suggested that the pre-hatching change in permeability of the membrane is caused by the action of chemicals produced by the larva. It is further suggested that the greater osmotic pressure of the fluid within the inner membrane is caused by external metabolites of the larva—either excretory or secretory.

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DARK-ADAPTING AND LIGHT-ADAPTING HORMONES CONTROLLING THE DISTAL RETINAL PIGMENT OF THE PRAWN PALAEMONETES VULGARIS[1]

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The first direct evidence that a light-adapting hormone is involved in the regulation of the distal retinal pigment of crustaceans was provided by Kleinholz (1936). He found that when extracts of the eyestalks of the prawn *Palaemonetes vulgaris* were injected into dark-adapted specimens kept in darkness, the distal retinal pigment approached the fully light-adapted condition. That this hormone is normally involved in retinal pigment migration was indicated by the fact that the eyestalks of dark-adapted specimens did not contain as much light-adapting hormone as those of light-adapted individuals.

Brown, Hines and Fingerman (1952) found a distal retinal pigment light-adapting hormone in the supraesophageal ganglia, circumesophageal connectives, and ventral nerve cord of *Palaemonetes vulgaris*. In addition, these investigators reported the presence in *Palaemonetes* of a distal retinal pigment dark-adapting hormone. Their evidence was indirect, having been based on differences in rates of dark-adaptation between control prawns and those injected with extracts of various organs, followed by transfer to darkness. No one has supplied direct evidence for such a hormone by causing the distal retinal pigment of a specimen of *Palaemonetes*, or of any crustacean, to approach the fully dark-adapted state while the specimens were kept under constant illumination (Knowles and Carlisle, 1956).

The aim of the present investigation was to provide direct evidence for a distal retinal pigment dark-adapting hormone in *Palaemonetes*.

MATERIALS AND METHODS

The prawns, *Palaemonetes vulgaris*, used in the experiments described below were collected in the Eel Pond at Woods Hole, Massachusetts. In the laboratory the stock supply of animals was kept in aquaria with running sea water.

The method used to determine the effects of tissue extracts on the distal retinal pigment was that devised by Sandeen and Brown (1952). The technique involves direct measurement of the position of this pigment in the living animal. The prawns were placed, one at a time, ventral surface down on the stage of a stereoscopic dissecting microscope. With the aid of an ocular micrometer and transmitted light (1) the width of the translucent portion of the compound eye in a plane parallel to the long axis of the eyestalk and (2) the length of the eye from the corneal surface to the dorsal pigmented spot at the base of the eye proper were measured.

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To render the distal clear portion of the eye more translucent and the proximal edge of this area more definite, the prawns were submerged in a dish of sea water on the stage of the microscope. The ratio of width of clear area (measurement 1) to total length (measurement 2) will be referred to as the distal retinal pigment index. Use of this ratio minimized the effect of size differences. In the fully dark-adapted eye the distal pigment abutted against the cornea; the distal pigment index was 0.00. In the fully lighted-adapted eye the distal pigment index was about 0.25. A typical ratio for a fully light-adapted eye was 10/40.

A magnification of 60× was used in the measurements. Each unit of the ocular micrometer at this magnification was equivalent to 24.4 μ. The distal pigment index of 10 prawns could be determined with ease in three minutes.

For all experiments the specimens were placed into black enameled pans containing sea water approximately 2.5 cm. deep. The pans were then exposed to an illumination of 20 ft. c. At this intensity the distal retinal pigment was about one-third of the distance toward the fully light-adapted position from the fully dark-adapted one. Under these conditions the distal pigment could respond to either light-adapting or dark-adapting hormone. Specimens with one eyestalk removed received the injections. Removal of one eyestalk resulted in the loss of an important source of retinal pigment light-adapting hormone (Brown, Hines and Fingerman, 1952). Presumably, therefore, one-eyed prawns would not be as readily able to antagonize any injected dark-adapting hormone as would intact specimens.

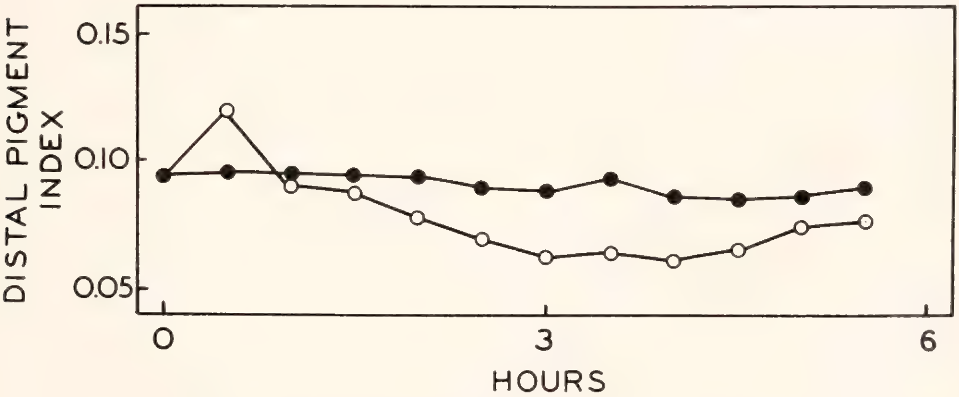

FIGURE 1. Responses of the distal retinal pigment to an extract of tritocerebral commissures, circles. Control, dots.

Extracts of eyestalks and of supraesophageal ganglia plus the circumesophageal connectives were prepared as follows. The organs to be assayed were extirpated and placed in sea water. When the desired number of each organ was available, the organs were transferred with a minimum of sea water to a glass mortar, triturated, and suspended in a sufficient volume of sea water to yield the desired concentration. When the extracts of sinus glands and optic ganglia were prepared, these tissues, because of their small size, were placed directly into mortars rather than into sea water. Every extract was assayed on 10 specimens. Control specimens were injected with 0.02 ml. sea water. All experiments unless otherwise stated were performed three times. Student's *t* test was used to determine the level of significance. The 95% level was taken as the minimal value for a significant difference between two means.

Experiments and Results

Influence of the tritocerebral commissure on the distal retinal pigment

Brown, Hines and Fingerman (1952) postulated that the tritocerebral commissure that runs posterior to the esophagus from one circumesophageal connective to the other contains little or no light-adapting hormone but does possess dark-adapting hormone. The first experiment was designed to test this hypothesis and to determine if the distal retinal pigment of specimens kept under constant illumination could be made to approach the fully dark-adapted state. A sufficient volume of an extract containing three tritocerebral commissures in each 0.02 ml. sea water was prepared. In Figure 1 are shown the results obtained when 10 prawns were each injected with 0.02 ml. of this extract. A transitory light-adaptation was produced that was followed by a dark-adaptation that lasted for several hours. This dark-adapting effect was highly significant statistically but the light-adaptation was not.

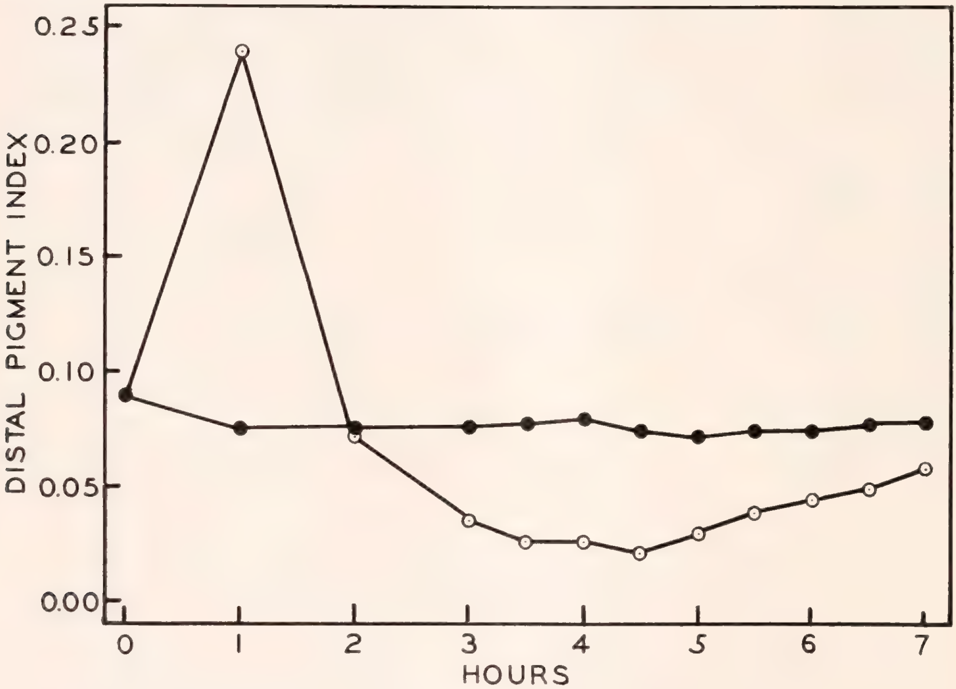

FIGURE 2. Responses of the distal retinal pigment to eyestalk extract, circles. Sea water control, dots.

The same experiment was also performed with one and two tritocerebral commissures per dose. In both of these experiments the distal retinal pigment became slightly more dark-adapted than the controls but the differences were not statistically significant. The data of these experiments are, therefore, not included herein.

Distal retinal pigment dark-adapting hormone in the eyestalk of Palaemonetes

The aim of this series of experiments was to ascertain whether a dark-adapting hormone is present in the eyestalks of *Palaemonetes*. For the first experiment of this group, eyestalks were extracted in a sufficient volume of sea water to yield a final concentration of one-third of a pair per 0.02 ml. This extract was injected into 10 specimens and its effect determined over a period of seven hours. Control specimens were also used. A strong light-adaptational response was observed. This was followed by a large dark-adaptational response. Because of the importance of this experiment it was done five more times. The data for the six experiments were averaged. The results are presented in Figure 2 where each point represents the mean of 60 individuals. These results are statistically significant.

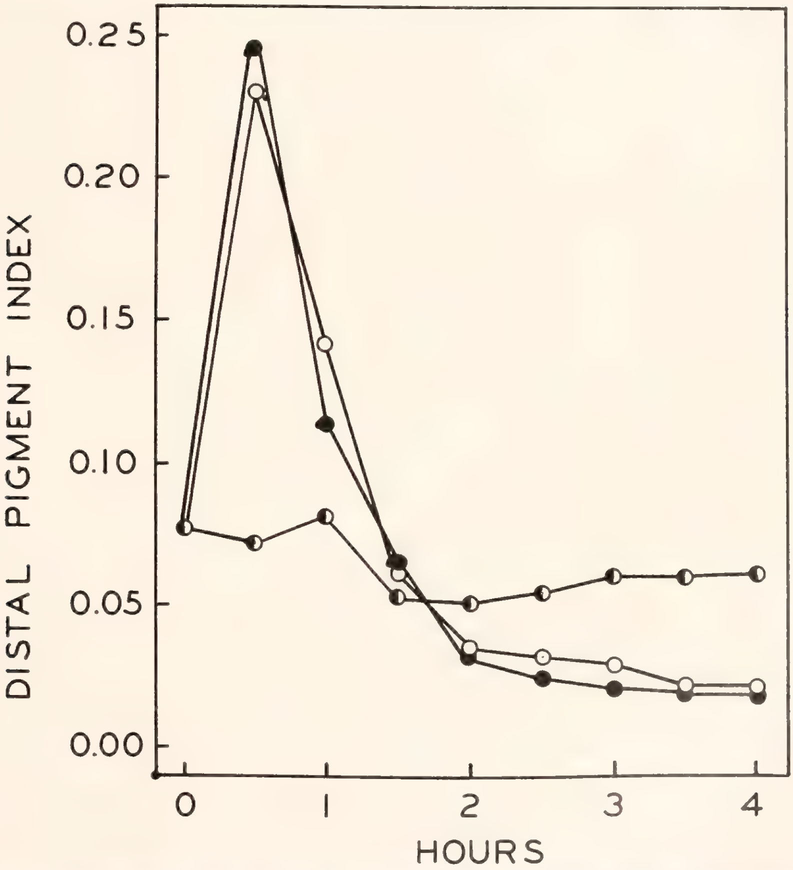

FIGURE 3. Responses of the distal retinal pigment to extracts of sinus glands (dots) and optic ganglia (circles). Sea water control, half-filled circles.

The sinus gland in the eyestalk of crustaceans is thought to be merely a storage and release center for neurosecretory products produced elsewhere, *e.g.*, in the optic ganglia (Knowles and Carlisle, 1956). The aim of the next experiment, therefore, was to determine whether the two retinal pigment hormones are found in the sinus glands and in the optic ganglia. These structures were dissected out, triturated, and suspended in sufficient sea water such that the final concentration was one-third of a complement per 0.02 ml. The experiment was performed three times with the same results. A light-adaptational response occurred that was followed by a dark-adaptational one (Fig. 3) just as was found with extracts of whole eyestalks (Fig. 2). The amplitudes of the responses shown in Figure 3 were slightly less than in Figure 2, presumably because of the decreased quantity of hormonal material in the extracts when the components of the eyestalks were separated from one another.

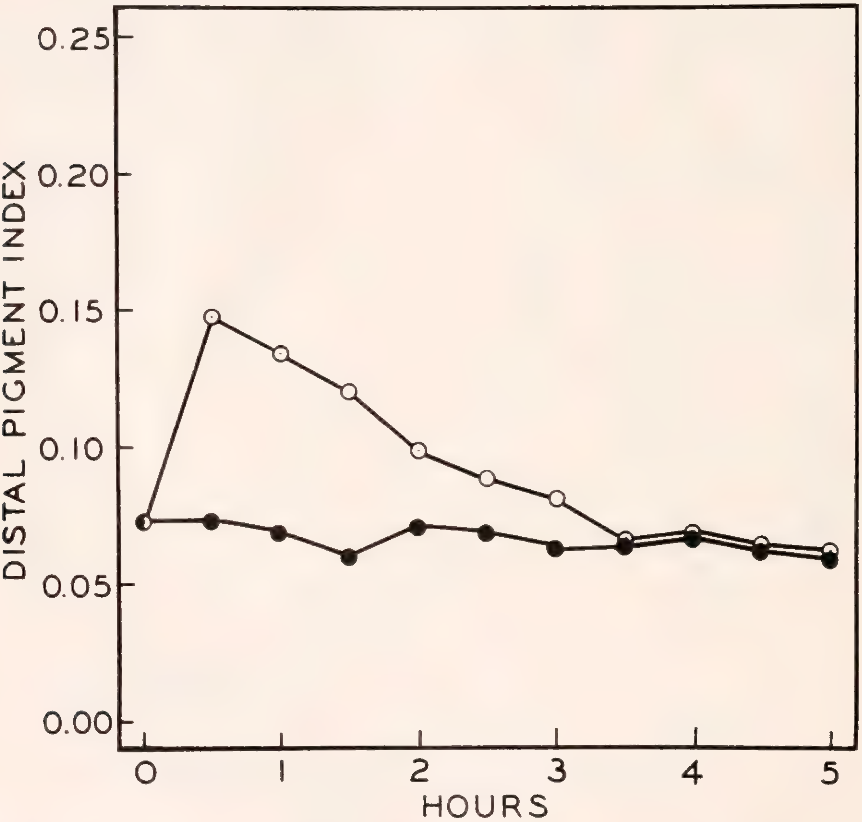

Figure 4. Responses of the distal retinal pigment to extracts of supraesophageal ganglia with the circumesophageal connectives attached from which the tritocerebral commissures had been removed (circles). Sea water control, dots.

The responses of the prawns to the extracts of sinus glands and optic ganglia were strikingly similar. Since the volume of the sinus gland is about one per cent that of the tissue in one eyestalk, the concentration of the hormones must be much greater in the sinus glands than in the optic ganglia.

An objection may be raised to the interpretation that the dark-adaptational response is due to a dark-adapting hormone, namely that the response is merely overcompensation on the part of the organism when removing the injected light-adapting hormone from the blood. To offset such an objection the final experiment was performed. Supraesophageal ganglia plus the circumesophageal connectives were dissected out. The tritocerebral commissures were then removed from these organs. These supraesophageal ganglia with the circumesophageal connectives attached were then extracted in sufficient sea water so that the final concentration was one-third of a complement per 0.02 ml. Such an extract would contain considerable light-adapting hormone in the virtual absence of a dark-adapting substance. The extract was then injected into 10 specimens. This experiment was also done three times. The averaged results (Fig. 4) revealed a statistically significant light-adaptational response and no dark-adaptation. If the dark-adaptational response shown in Figures 2 and 3 had been merely overcompensation then it would have occurred here also.

DISCUSSION

The results presented herein provide direct unequivocal evidence for a distal retinal pigment dark-adapting hormone in *Palaemonetes*. The indirect evidence for this endocrine factor presented by Brown, Hines and Fingerman (1952) finds support in these experiments. The results represent the first time that dark-adaptation has been induced in light-adapted specimens kept under constant illumination.

The dark-adapting hormone appears to be subordinated to the light-adapting one, being able to function only after the latter hormone has run its course. However, the effect of the dark-adapting hormone persists much longer than that of the light-adapting substance (Figs. 2 and 3).

The presence of these antagonistically functioning hormones probably provides *Palaemonetes* with more precise control of the position of its distal retinal pigment than it would have if these prawns produced light-adapting hormone alone. The prawns can secrete an antagonist when the pigment must be moved rapidly to the dark-adapted state rather than be forced to wait for the light-adapting hormone to be eliminated from the circulation. As information is being gathered about endocrines in crustaceans, we find more instances where processes are controlled by oppositely functioning substances. Such was also the case with the red chromatophores of *Palaemonetes*. Brown, Webb and Sandeen (1952) demonstrated red pigment concentrating and dispersing hormones in this prawn where only the concentrator had been found previously.

SUMMARY AND CONCLUSIONS

1. The distal retinal pigment of the prawn *Palaemonetes vulgaris* is regulated by light-adapting and dark-adapting hormones.

2. These hormones are found in the sinus glands and central nervous organs.

3. The dark-adapting hormone was demonstrated by inducing with tissue extracts dark-adaptation of the distal retinal pigment of light-adapted specimens

maintained under constant illumination, the first time this has been accomplished in any crustacean.

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HISTOPHYSIOLOGY OF GILL AND KIDNEY OF CRAB OCYPODE ALBICANS

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Brachyuran crabs possess the ability to regulate the internal level of the chloride ion against shifts in the external level of this ion. Webb (1940) and Jones (1941) showed that this ability varies in different species of crabs, and becomes functional over an increasingly wider range as the animals move away from the sea into an estuarine or shore habitat. Data presented by Flemister and Flemister (1951) indicated that the ghost crab, *Ocypode albicans* (Bosq), is able to regulate the internal chloride ion against a hypotonic environment of 200 millimoles of chloride per liter, and a hypertonic environment of 600 millimoles per liter. That is, within this range of environmental chloride ion, the internal chloride ion of the crab is maintained at 375 millimoles per liter. Such regulation is accomplished by reciprocal mechanisms for uptake and loss or absorption and secretion of the chloride ion, these mechanisms being located in cells which occupy appropriate sites in reference to the external and internal environments.

The principal site of chloride ion uptake by decapod Crustacea is thought to be the gills. The uptake of ions from the environment by fresh water animals was reported by Krogh (1937) as a probable function of the gill. Webb (1940) suggested that the histology of the gill of *Carcinus maenas* was compatible with the process of salt and water transfer. Isolated gills of the crab *Eriocheir sinensis* were shown to absorb ions from the environment by Koch, Evans and Schicks (1954). Chloride ions are excreted from the body by the kidney or antennal gland. It has been established that under conditions of excess chloride ion in the environment, the urine secreted by the kidney contains a higher proportion of chloride ion than it does under conditions of low chloride ion in the environment. An investigation of the relation of oxygen consumption to chloride ion regulation reported by Flemister and Flemister (1951) led to the conclusion that chloride ion regulation by the kidney was supplemented by the activity of some other tissue or tissues. Excretory cells are found in the gill and in the hepato-pancreas, in addition to the kidney. Early accounts of crustacean anatomy by Cuénot (1895) and Pearson (1908) describe these cells and attribute an excretory function to them. A more recent study by Lison (1942) emphasizes their possible excretory role. These findings suggest that gill tissue may be active in chloride ion loss, as well as uptake. The hepatopancreas is likewise a site at which chloride ion may be absorbed or excreted, although its role in regulation is doubtful. Travis (1955) has described the functional histology of this structure in detail. Similarly, the tufts of branchial epithelium

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which partially line the gill chamber of *Ocypode albicans* occupy a position in which they might contribute to the transfer of ions and water. An investigation of the histophysiology of gill, kidney and branchial epithelium of *Ocypode albicans* was undertaken with the special objective of examining their role in the transfer of the chloride ion.

METHODS

Ocypode albicans was collected in the summer months on the beaches at Rehoboth, Delaware, and at Bermuda in March and April. The animals were brought into the laboratory where they were maintained on damp sand and offered food until they were subjected to experimental treatment. The entire period of their stay in the laboratory was not longer than ten days or two weeks. No animals showed indications of impending molting and none molted in the laboratory.

Animals were acclimatized in sea water containing, respectively, 200, 400 and 600 millimoles of chloride per liter. The chloride ion level of the blood of *Ocypode albicans* is maintained in the range of 375 to 400 millimoles of chloride per liter; thus the range of salinities was hypotonic, isotonic and hypertonic in reference to the internal chloride ion concentration. Animals remained in the experimental tanks for seventy-two hours. Blood and urine were collected and analyzed for chloride ion content before and after acclimatization, using methods described in a previous paper (Flemister and Flemister, 1951).

At the end of the period of acclimatization, tissues were removed for study. Tissues taken included gill and antennal gland, which were fixed without further dissection, and the branchial epithelium. This latter tissue in *Ocypode albicans* is the tufted lining of the inner face of the branchial chamber. It was removed, cut into two portions and these fixed flattened out. Fixation in formalin or Bouin's fixative, followed by hematoxylin and eosin or Mallory's connective tissue stain, was used for general histological examination. Regaud's fixative followed by post-chroming resulted in fixation of mitochondria which were then stained by iron hematoxylin or Altman's acid aniline fuchsin.

The Leschke method for the detection of chlorides was used according to the sequence described by Copeland (1948). Tissues were removed from the animal and fixed, without washing, in one per cent silver nitrate made acid with nitric acid. This fixation was accomplished in the dark, as was the development in Eastman D-11 (diluted 1:4) and final fixing in Eastman F-5 (diluted 1:5). The tissues were then washed, dehydrated, embedded in paraffin and sectioned. Sections were gold toned and sometimes counter-stained with eosin. The picture of silver deposition which resulted, as will be discussed below, led to testing for a clue as to the nature of the material reacting with the silver. Polyphenols, urates and fats might possibly be expected to react with the silver in the procedure described. These were individually tested for by the following procedures.

The Hollande method for the detection of urates was used as described by Glick (1948). This involved fixation in one per cent silver nitrate solution in neutral formalin, in the dark. Polyphenols were tested for by treating sections of formalin-fixed material in ammoniacal silver nitrate in the dark by Masson's method (Lison, 1936). Reduced silver deposits in each of these indicates presence of material tested for. Some formalin-fixed tissues were washed and imbedded in Carbowax after the method of Blank and McCarthy (1950) and the sections stained with Sudan III for the detection of fats.

RESULTS

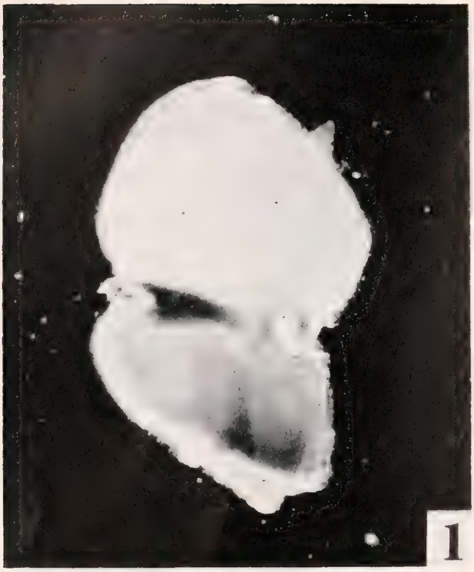
The gill

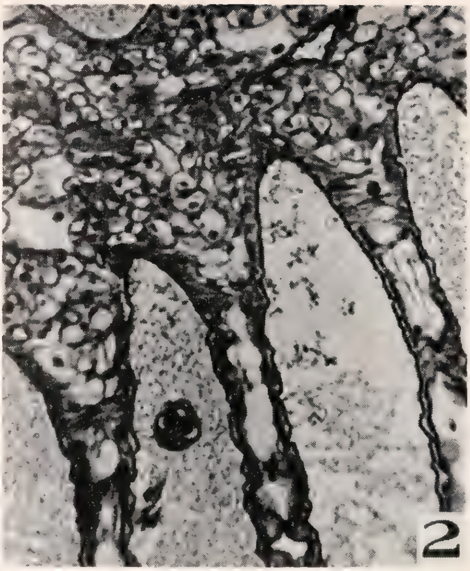
Descriptions of the histology of decapod crustacean gills are to be found in the paper of Cuénot (1895) and the monograph on *Cancer* by Pearson (1908). The gill of *Ocypode albicans,* as that of other brachyurans, is formed of a number of lamellae, or broad flattened plates arranged serially in pairs along a central gill stem (Fig. 1). The gill stem provides support for the lamellae and is the pathway for the afferent and efferent branchial vessels. The entire outer surface of the gill is covered by a thin layer of chitin which is about 1 μ in thickness.

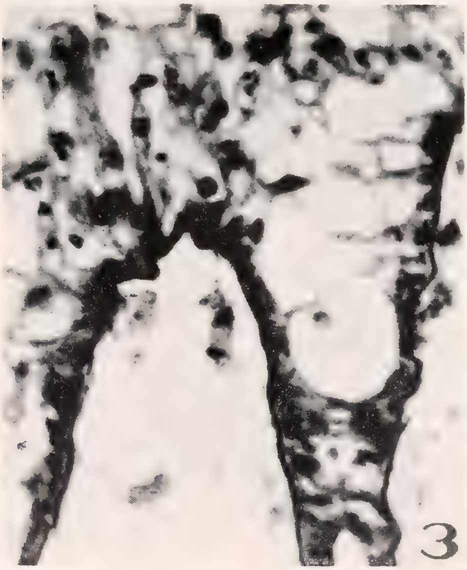
The individual leaflet or lamella may be likened to a flattened thin-walled sac. Underlying the chitin is a continuous lining of epithelial cells (Fig. 2). At irregular intervals the faces of the lamella are joined by large cells whose cytoplasm contains distinct fibrils, and which constitute pillar cells. The distal border of the lamella is expanded, being free of pillar cells and traversed by an occasional connective tissue fiber. The irregular cavity within the lamella resulting from this arrangement of pillar cells and fibers is filled with blood in life and an occasional blood cell is seen in sections. The lamellar blood space communicates with the afferent and efferent branchial vessels. The epithelial cells of the lamellae are continued as the lining of the gill stem. Collagen fibers of some thickness are found in the stem itself. Large connective tissue cells conforming to the classification of Leydig cells of the first order, according to Kükenthal (1926–1927), compose the chief support of the gill stem. Smaller spindle-shaped Leydig cells of the third order may also be found. Blood cells are commonly seen lying in the interstitial spaces.

In addition to the cells described, there is yet another type which appears to be unique to the gill. It was termed a branchial excretory cell by Cuénot (1895) and a branchial athrocyte by Lison (1942). These cells are large, oval in shape, with the cytoplasm arranged in a peripheral layer surrounding a vacuole. The nucleus is displaced to one side, close to the cell membrane. The vacuole contains material which in fixed sections appears as an aggregate of granules incompletely filling the space. The cells are arranged in irregular rows or aggregates which protrude into and are bathed by the blood which passes through the stem. Lison (1942) and earlier investigators have observed that certain classes of dyes injected into the blood stream may be accumulated by these cells and from this function is derived their classification as branchial excretory cells.

In mitochondrial preparations it is immediately apparent that the lamellar epithelium is rich in mitochondria, while the branchial excretory cells show a sparse or absent population (Fig. 3). It is also apparent that although the lamellar epithelium is continuous with the lining of the gill stem, there is a marked decrease in the mitochondrial count in the cells lining the gill stem. The mitochondria may be filamentous or arranged in minute rows of granules; they occupy the cytoplasm of the cell on either side of the nucleus, and do not appear to be constantly located either toward the base or toward the distal surface (Fig. 4). In preparations made







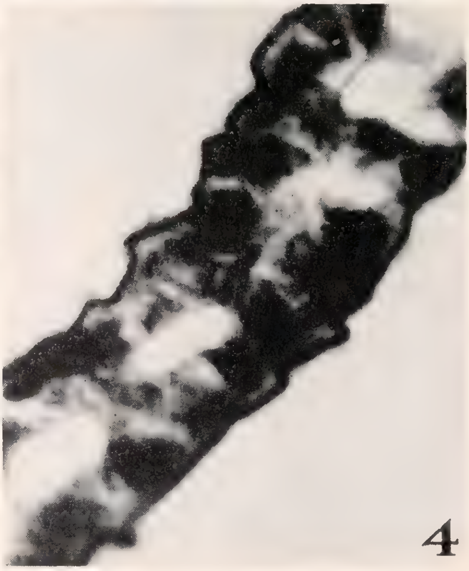


FIGURE 1. Portion of gill. 17 ×. Several lamellae attached to central stem. Afferent, ventral and efferent, dorsal, vessels appear as light, roughly circular areas. Darker part of stem is area of branchial athrocytes.

FIGURE 2. Longitudinal section of stem of gill with several lamellae. 200 ×. Note loosely packed athrocytes, blood spaces and occasional connective tissue fibers in stem. Lamellae are lined by epithelium below a thin chitin cover. Pillar cells appear to join faces of lamellae.

FIGURE 3. Portion of stem and lamella stained with iron hematoxylin. 380 ×. Mitochondria show as dark clumps in lamellar epithelium. Athrocyte in extreme left center field shows enclosed material lightly stained. Very dark cells in stem are blood cells.

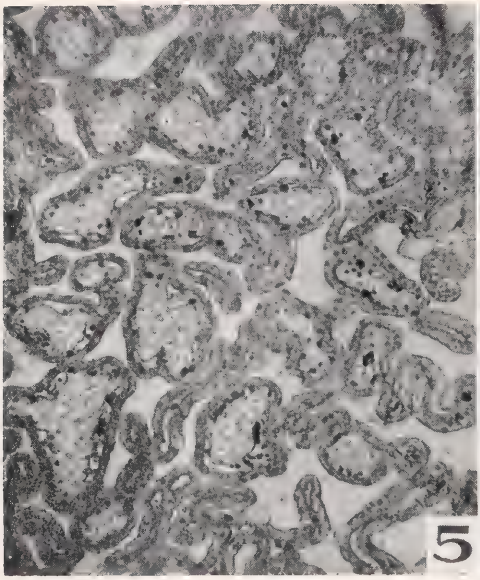
FIGURE 4. Section of lamella, iron hematoxylin. 860 ×. Mitochondria appear as dark clumps and threads. Chitin covering heavily stained.

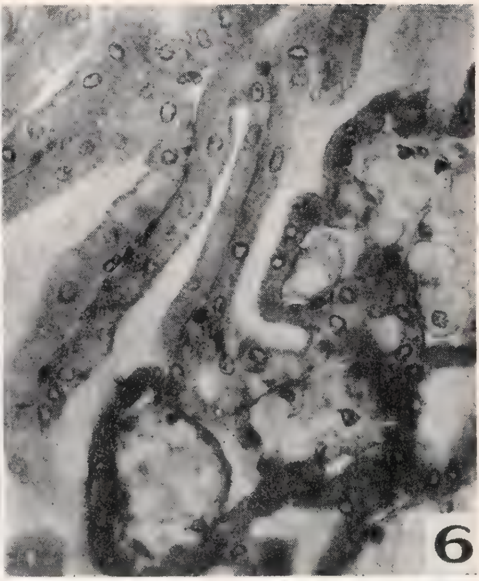
from crabs acclimatized to 200 millimoles of chloride per liter the lamellar cells show some vacuolation; in such instances the mitochondria line up at the borders of the vacuoles. Vacuolation is less in lamellar cells of material taken from crabs which had been living in sea water containing 400 and 600 millimoles of chloride per liter, but otherwise the picture is the same. Mitochondria appear as scattered granules in the peripheral cytoplasm of the branchial excretory cells. However, the material present in the vacuoles of these cells often stained with the mitochondrial stain.

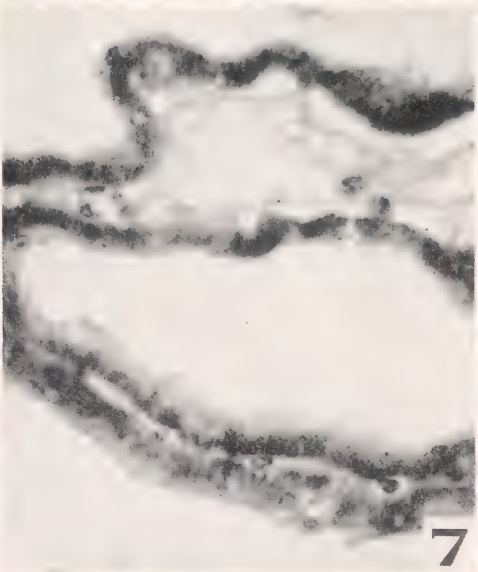
The Leschke test for the detection of chlorides gave clear results on one point: there is never, under any circumstances, any evidence of silver deposition in the lamellar epithelium, the epithelium of the stem, any of the connective tissue of the stem, or in any of the blood cells. There is blackening of the covering chitin, as would be expected since the tissues were not washed before fixing. There is occasional outlining of the nucleus, and connective tissue fibers may show blackening. In all of the tissues prepared from Rehoboth Beach crabs there is blackening of the material contained within the vacuole of the branchial excretory cells. This is not uniformly true of the crabs taken in Bermuda. Careful examination of material taken from crabs from the three classes of environmental situations shows no obvious quantitative differences which might reflect activity in the regulatory mechanism.

The interpretation of the occurrence of deposits of reduced silver as evidence of the localization of chloride is open to dispute. The Leschke method depends upon the formation of silver chloride after treatment with acid silver nitrate and subsequent reduction of silver by means of a quinone-containing solution. Such a method was meticulously explored by MacCallum (1905) using a variety of biological materials. It was his conclusion that only halides of all substances in biological materials would give this particular reaction. It is to be noted that in his experiments, proteins and other substances used were purified by repeated treatment to rid them of all free chloride. His conclusions have been used by a number of workers to justify their results with the Leschke method. Keys and Willmer (1932) used this method in determining the location of the "salt cells" of fish.

There are two lines of objection to a blanket acceptance of silver deposits as evidence of the localization of chlorides. One of these is based on the highly diffusible nature of the chloride ion which tends to move freely in biological fluids and across membranes. Silver is a heavy metal and tends to be adsorbed upon membranes so that penetration of the solution may be uneven. Thus, any reduced silver found after the treatment is complete may, or may not, represent a true localization of chloride present *in vivo*. The second objection, which in some respects is more serious, is that certain substances found in tissues are capable of immediately reducing acid silver nitrate in the dark. The best known of these is Vitamin C, ascorbic acid, and this property is the basis of the method of Giroud (1938) for determining the location of Vitamin C in tissues. Polyphenols give a silver precipitate after treatment with ammoniacal silver nitrate, and urates yield a silver precipitate after treatment in neutral silver nitrate. Tissues from *Ocypode albicans* subjected to these tests showed no evidence of reduced silver in the locations observed after the Leschke test or in any other locations. Vitamin C is regularly found in the cytoplasm of the cell, occupying a position in the neighborhood of the Golgi apparatus. Thus the nature of the material contained within the vacuoles of the branchial







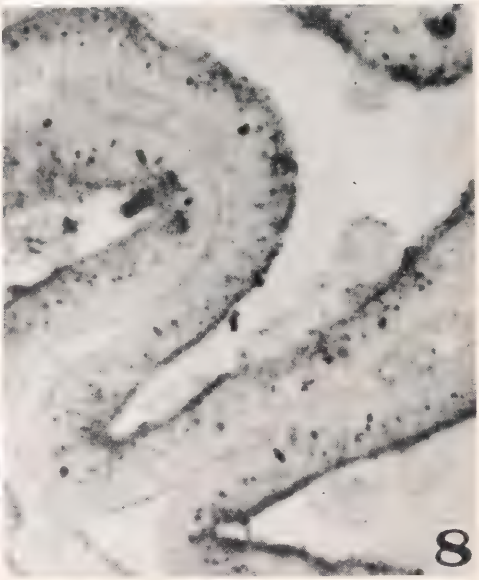


FIGURE 5. Section of kidney, hematoxylin and eosin-stained. 100 ×. Typical view of labyrinth, showing renal tubule cells surrounding saccule cells. The latter are very pale. Occasional very dark cells between the two layers are blood cells in the blood spaces.

FIGURE 6. Labyrinth, hematoxylin and eosin. 380 ×. The lower left hand portion of the picture shows renal tubule cells. Note brush border, and the secretion bleb being extruded into the lumen. In the upper right hand corner are a few saccule cells, very pale and appearing almost empty.

FIGURE 7. Labyrinth, iron hematoxylin. 380 ×. At top renal tubule cells surround saccule cells which have not been stained by iron hematoxylin. Section of renal tubule at bottom shows mitochondria situated towards the hemocoele, in the basal portion of the cells.

excretory cells is still undetermined. It seems reasonable to speculate, however, that under some circumstances this might be a complex molecule which is capable of combining loosely with chloride. An alternate interpretation would be that it is a molecule which under some circumstances carries a reducing radical of yet undetermined nature. The material is not fat: it persists after normal dehydration and clearing procedures; it stains with cytoplasmic stains generally; it does not stain with Sudan III after Carbowax embedding and sectioning. What it is remains undetermined; that the reduced silver indicates the location of a chloride remains to be positively substantiated.

The kidney

The histology of decapod crustacean kidneys is amply treated in the descriptive works of Marchal (1892) and Pearson (1908). The kidney of *Ocypode albicans* conforms to these descriptions. The labyrinthine structure is the result of the growth in close proximity of two sac-like portions of the excretory tubule. The floor of the more dorsal end sac pushes into the roof of the more ventral renal tubule with a consequent close interdigitation of the layers. The lumen of the end sac communicates with the lumen of the renal tubule which in turn empties through a bladder to the outside. There is no direct connection between the hemocoele and the lumen of the excretory apparatus; all materials eliminated must pass through the cells either of the end sac or renal tubule.

Typical sections through the kidney show portions of the end sac, renal tubule, and areas of interdigitation (Fig. 5). Comparisons of end sac epithelium and renal tubule epithelium can be made easily in the areas of interdigitation, at which locations the end sac epithelium always constitutes the inner layer of cells, surrounded by an outer layer of renal tubule epithelium. The appearance and staining capacities are sufficiently different so that renal tubule cells may always be distinguished from end sac cells. Renal tubule cells are cuboidal in shape, stain deeply and have a well defined brush border on the surface of the cell facing the lumen (Fig. 6). In contrast the cells of the end sac are large oval or cuboidal cells generally arranged in one layer although they may occasionally form two indistinct layers. A considerable portion of the cell is occupied by a vacuole which may contain granular material, and the nucleus is consequently displaced to one side. Staining is invariably light or pale in contrast to the deeper staining renal tubule cells. The end sac cells resemble closely the branchial excretory cells described for the gill, and they have been termed kidney athrocytes by Lison (1942).

Mitochondria are found in renal tubule cells, usually as filaments occupying the area of the cell towards the hemocoele (Fig. 7). There are generally few or no mitochondria observed in the end sac cells, and the material of the vacuole does not stain with the mitochondrial stain.

Kidney tissue treated by the Leschke method shows silver deposition as follows. The contents of the vacuoles of the end sac cells are blackened. The degree of blackening is not uniform, and shows no correlation with the observed regulatory

FIGURE 8. Labyrinth, silver fixation. 860 ×. Portion of renal tubule from crab acclimatized to hypertonic environment. Heavy deposition of silver apparently in brush border area of cell, and outlining secretion blebs. Note that silver also accumulates within cell in discrete particles. They are not nuclei, and significance of their occurrence is not known.

activity of the animal. Tests similar to those made on gill tissues for polyphenols, urates and fats gave negative results. The nature of this material is undetermined. In kidney tissue removed from crabs acclimatized to 600 millimoles of chloride per liter there is a pronounced deposit on the lumen side of the renal tubule cell (Fig. 8). This blackening appears to involve the brush border as well as the immediately adjacent lumen edge of the cell. The blebs of secretion characteristic of this type of cell are also outlined with faint depositions of silver. The cytological picture here coincides with the known physiological activity of the kidney, which is excretion of excess chloride under the conditions of regulation to the hypertonic environment. There seems to be little doubt that the site of excretion is through the renal tubule cells. Silver deposition in kidneys taken from animals acclimatized to 200 millimoles of chloride per liter shows along the lumen border of cells, but not outlining the secretion blebs; the silver always seems to be within the cell. In several specimens from Bermuda, the sections show no silver except at the blood side of the cell. This was not observed uniformly in the tissues of crabs acclimatized to a hypotonic environment. Under such conditions the kidney is excreting a dilute urine with reference to the chloride content and it is perhaps impossible to detect accumulation or reabsorption of chloride which might be occurring.

The branchial epithelium

The lining of the inner surface of the gill chamber is the branchial epithelium, which in *Ocypode albicans* is developed into numerous tufts which presumably offer increased surface for respiratory exchange. The membrane is composed chiefly of a large blood space surrounded by the stellate and spindle-shaped connective tissue cells characteristic of Crustacea. The main blood channel protrudes into finger-like projections which follow poorly defined ridges. Within the network of connective tissue are scattered large cells corresponding to the reserve cells of Cuénot (1895). These cells have the property of accumulation and storage of proteinaceous materials. The outer covering is composed of a single layer of flattened epithelium covered by a very thin layer of chitin.

None of the cell types show any evidence of possible active absorption or secretion. The mitochondrial picture does not indicate such activity on the part of any cells; there are no athrocytes; and there is no evidence of tegmental glands associated with this epithelium. It seems probable that respiratory exchange occurs across this membrane, although the contiguity of external and internal environments is not as close as in the gill lamellae. Although salt transfer, and that of water, could be accomplished by direct osmotic forces it does not seem possible that this membrane takes an active part in salt and water regulation.

DISCUSSION

The microscopic anatomy of the gill and kidney of *Ocypode albicans* can be related to the function of transfer of chloride ion between the blood of the animal and the environment. The hepato-pancreas and the branchial epithelium are likewise areas at which transfer may occur, but these sites do not appear to be concerned with regulation. Gill and kidney on the other hand are not only areas of passive transfer, but are also elements of the regulatory mechanism. The regulatory mechanism has been demonstrated by Flemister and Flemister (1951) to be effective in *Ocypode albicans* over a hypotonic and hypertonic range of environmental chloride ion content, and it has been further demonstrated that this regulation requires the expenditure of energy.

The chief portal of entry of the chloride ion into the body of the crab is at the gill surface. The lamellar cells which constitute the cellular surface of the gill are closely associated with the blood stream, which bathes one surface, and the external environment which bathes the other surface. The cells give evidence of being active in some secretion or absorption process by their rich population of mitochondria, which is a generally accepted sign of a metabolically active cell. The lamellar cells under no circumstances showed any evidence of accumulation of chloride ion, and it is assumed that the absorption process is a continuing one and does not involve even temporary accumulation within the cell. These same cells are the final route of carbon dioxide as it leaves the body. It is known from the work of Ferguson, Lewis and Smith (1937) that the gills of crustaceans contain a large amount of the enzyme carbonic anhydrase as compared with the blood or other tissues taken from the animals. The diagram (Fig. 9) shows how the excretion of carbon dioxide may be related to the function of chloride ion uptake by the gill of the crab. Carbon dioxide from the body tissues arrives at the lamellar cell in the form of bicarbonate, and is transferred across the cell to the environment. As bicarbonate leaves the cell, chloride ion enters, maintaining the ionic balance; similar exchange occurs at the blood surface of the cell, with the net result that chloride ion enters the blood stream as carbon dioxide is lost from the body. This uptake is augmented by some specific cellular activity which results in the production of carbon dioxide which is removed from the cell as bicarbonate produced by the activity of carbonic anhydrase. It is believed that this absorption mechanism is working under all conditions of tonicity of the environment, and is independent of the ion content of the environment. It

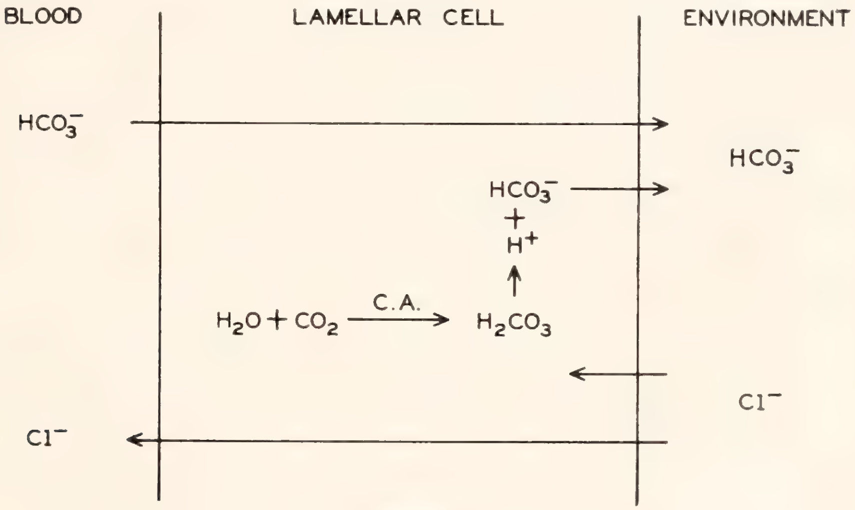


FIGURE 9.

is effective in supplying enough chloride ion to maintain the internal level until the external level falls below about twenty-five per cent of the internal level (Flemister and Flemister, 1951). Below this level it is still working as can be observed from the uptake experiments cited above. When the crab is subjected to a hypertonic environment, the mechanism is still at work, and supplements the osmotic force tending to drive chloride ion into the body. The internal chloride ion level then is maintained by excretion of excess chloride ion by the kidney, and perhaps at other sites. It is interesting to note that in *Gecarcinus lateralis* as reported by Flemister (1958) the blood chloride ion level tends to rise above the normal level when the crab is living in hypertonic environment. It appears that the excretory mechanism cannot keep up with the intake in this particular situation.

The renal tubule cells of the kidney are the principal sites of chloride ion excretion. The cells lie between the blood stream and the lumen of the kidney which communicates with the exterior; they show the brush border and mitochondria characteristically associated with absorbing or secreting cells; it is reported by Krugler and Burkner (1948) that alkaline phosphatase is found in these cells; and the cytological picture resulting from the Leschke test adds evidence that chloride ion may be excreted from the body at this site. Analysis of the urine of crabs reported by Flemister and Flemister (1951) showed that there is always chloride ion in the urine, and the amount increases directly with increasing chloride ion in the environment. The mechanism of secretion is not known, but it appears to be a function of the renal tubule cells. There is no evidence here that these cells can reabsorb chloride ion from the urine. Hence the renal tubule cells are the route of chloride ion out of the body.

The athrocytes of the end-sac and the branchial athrocytes are concerned with the removal of large, poorly diffusible molecules from the blood stream. According to Lison (1942) this is accomplished by a process of accumulation, the exact nature of which is not understood. Final removal from the body results from the breaking away of the end-sac athrocytes so that they float freely in the lumen of the kidney and are lost from the body with the urine. There is no such obvious final route for the gill-stem athrocytes, and they may perhaps be looked upon as analogous to the fixed macrophages of the vertebrate reticulo-endothelial system. However, it should be noted that specific evidence for a phagocytic action is lacking. It is difficult to imagine how the athrocytes could be involved in the mechanism resulting in chloride ion regulation, unless the ion is somehow attached to a large poorly diffusible molecule within the cell, and thus removed from the blood stream. If the Leschke test is assumed valid as an indicator of the presence of halides, then the athrocytes contain halides in quantity far greater than other cells. There is no quantitative difference in the amount of halide bound by the athrocytes of crabs taken from hypotonic or hypertonic environments. Perhaps the only function of these cells is accumulation, and there can be no eventual release back into the blood stream. These cells would then act antagonistically to the lamellar cells which are continually absorbing chloride ion from the environment, and they would supplement the function of the renal tubule cells. It is to be noted that this speculation is based on an assumed validity of the Leschke test.

There is some indication that the kidney is not the only site of chloride ion loss from the body, according to Flemister (1958). Granting that the role of the athrocytes is hypothetical, one other source of leaking of chloride ion could be at the branchial epithelial surface. Since this surface is relatively thin and lies between blood stream and environment, there is ample opportunity here for exchange of ions as a result of osmotic differentials. Thus the branchial epithelium might serve as a portal of entry of chloride ion in a hypertonic medium, a site of chloride ion loss in a hypotonic medium. The regulation of the blood chloride ion level must depend on those cells which are active in absorbing or secreting chloride ions, those of the lamella of the gill and the renal tubule of the kidney.

Summary

1. The fine structure of the gill and kidney of *Ocypode albicans* was examined for evidence of participation in the transfer of water and the chloride ion.

2. The epithelium of the gill lamellae was found to have the characteristics of a secreting epithelium. No other cells associated with the gill structure had either the position or morphology to be considered important in this function. The athrocytes of the gill stem probably do not participate in salt-water regulation, but are concerned principally with the removal of poorly diffusible ions from the blood stream. No mechanism of such removal can be interpreted from the present study.

3. The cells of the renal tubule of the kidney were found to have the characteristic brush border and mitochondrial picture associated with actively secreting or reabsorbing epithelia. Silver deposition following the Leschke test gave evidence that these cells are involved in the excretion of chloride, and may possibly also act to reabsorb chloride from the urine. The athrocytes of the kidney end-sac, like those of the gill stem, probably do not function in salt or water regulation. Materials accumulated within these cells are lost to the body when the cells break away and float free in the urine.

4. Absorption of salt from the environmental medium is accomplished by an energy-using mechanism in the lamellar cells. This is a constant function and is associated with the carbonic anhydrase mechanism working in the excretion of carbon dioxide. Excretion of salt by the renal tubule is probably also a constant function, although no evidence as to its possible mechanism is available. Reabsorption of salt at this location is a possibility; the triggering mechanism is probably a falling chloride concentration in the blood.

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ORGANIC PRODUCTIVITY IN THE REPRODUCTIVE CYCLE OF THE PURPLE SEA URCHIN[1]

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The gonads of a gravid purple sea urchin (*Strongylocentrotus purpuratus*) may contribute as much as one-fifth to the total wet weight of the animal. On the other hand, the shrunken gonad of an immature animal or one which has recently spawned may be only one-eighteenth as large. The development of the gonad represents a remarkable synthesis of organic material, since the larger part of the protoplasm of a sea urchin is gonadal during the breeding season, the only other organ of any bulk being the intestine. The intestine in turn owes part of its bulk to its food contents, the epithelium itself being quite delicate. The volume of perivisceral fluid bears an inverse relation to the gonads, being present in larger amounts when the gonad is less well developed. The perivisceral fluid, however, contains little organic material (Lasker and Giese, 1954). Furthermore, its organic constituents do not vary in any striking or systematic way during the year (Bennett and Giese, 1955). A fairly good measure of organic productivity in the sea urchin might therefore be gained by a study of the increase in organic constituents in the gonads during their growth from immature (or spent) to gravid condition. The results of such study are reported in this paper.

METHODS

For most of the experiments reported here, sea urchins were collected at the monthly low tide at Yankee Point, near Carmel, California. In a few instances specimens were obtained near Moss Beach, California. The gonad index was determined for each of ten specimens, the index being the ratio of the volume of gonad to wet weight of animal, times 100. The total nitrogen (TN), non-protein nitrogen (NPN), lipid, and glycogen contents of samples of gonad were determined. For one male and one female, water and ash content of the sample were also determined monthly. From samples at the height of the season, and also after the spawn-out, determinations were made of the desoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as lipid, total nitrogen and non-protein nitrogen at the same time, and in a few samples reducing sugar (RS) content was determined. These data give a biochemical picture of the constituents of the gonads correlated with the gonadal cycle over an entire year.

For the biochemical determinations on the gonads of each animal, several samples of various wet weights (several grams) were placed in a vacuum desiccator over concentrated sulfuric acid and dried for about 12 hours. When tissues were to be used for glycogen analysis, a few drops of 10 per cent trichloracetic acid were injected before drying to prevent glycolysis by enzymes during the drying process. All analyses were done in duplicate; the duplicates varied by only a few per cent.

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For determination of total nitrogen, a given sample was digested in sulfuric acid with selenium catalyst over electric heat or gas flame, and from an aliquot of the digest the ammonia was distilled with a Markham still (Markham, 1942), or in a Conway diffusion cell (Conway, 1947), into borate buffer containing brom-cresol green and methyl red as indicators. The borate was then titrated to the original color with 0.01 N sulfuric acid. Usually several weights of samples were tested and to one of them a known weight of a nitrogen-containing compound (glycine) was added to serve as a check on the accuracy of the method.

For determining non-protein nitrogen 1 ml. of 10 per cent trichloracetic acid (TCA) was added to a 10–30 mg. sample of gonad and the tissue was macerated with a glass rod. It was heated to 80–100° C. in an oven for 15 minutes, allowed to cool, centrifuged, and the supernatant plus two washings of the precipitate were added to the flask which was then placed on the digestion rack and the nitrogen content determined as described above. The non-protein nitrogen subtracted from the total nitrogen is taken to give the protein nitrogen (PN) [3]. This is multiplied by the factor 6.25 to convert to protein.

Total lipids in gonadal tissue were determined by extracting 100-mg. samples with 10 ml. ethyl ether in a micro-Soxhlet apparatus, refluxing being continued for two hours. Many samples were extracted at the same time on a sand bath.

Glycogen was determined in the following way (Good *et al.*, 1933; Meyer, 1943). The ground dry sample was treated with an equal volume of 10 per cent TCA, cooled, and the supernatant was transferred to a lusteroid tube to which was added 1 ml. of distilled water wash of the precipitate. After addition of 2.5 ml. of 95 per cent ethanol and mixing, the sample was centrifuged and the supernatant fluid was discarded and the tube allowed to drain for several minutes. To it was added enough warm water to give about 70 μgm glycogen per ml. and the content of glycogen was determined by the anthrone method (Seifter *et al.*, 1950). Reducing sugar was determined in the supernatant fluid of a homogenized gonad by the Somogyi method (1945; 1952) which involves first the precipitation of the protein by TCA, centrifuging the sample, and testing of the supernatant solution.

Water content was determined by weighing minced tissue before and after drying in the desiccator over sulfuric acid. Ash content was determined on a known dry weight of gonad (about 100 mg.) heated to 450–500° C. in a porcelain crucible for three to eight hours.

Nucleic acids were extracted using the Hershey, Dixon and Chase (1953) adaptation of the Schmidt-Thannhauser (1945) and Schneider (1945) procedures, acid-soluble phosphorus being removed by cold 10 per cent TCA, phospholipid being removed with ethanol and a mixture of ethyl ether and ethanol (60° C.). RNA was removed with KOH, DNA being precipitated with 5 per cent TCA (Leslie, 1955). The indole reaction of Ceriotti (1952) was used for DNA and the orcinol reaction of Ogur and Rosen (1950) was used for RNA; the details of the method as used here have been described elsewhere (Iverson and Giese, 1957). Some studies were made determining the nucleic acids by the phosphorus method (Fiske and Subbarow, 1925) but they were considered less reliable and are not reported here.

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[3] Although it is classical procedure, some question exists whether this is entirely justified here, because when a direct test for protein nitrogen is made on the residue remaining after extracting acid-soluble phosphates, phospholipids and nucleic acids from the tissue mash, only about a half to a third as much is obtained as by the difference between total nitrogen and non-protein nitrogen. It is possible that some of the proteins are dissolved by the extraction procedures, but additional studies are desirable.

RESULTS

The average values for some chemical contituents of gonads of male and female sea urchins taken each month of the year 1956 are given in Table I. Certain trends appear in the data of this table. At times of the highest gonad index, the gonads per unit weight tend to contain more lipid, protein, glycogen and ash and less water (especially in the female) than at the time of low gonad index. A more significant rendition of the data of Table I is given in Figure 1, because it shows the distribution of each chemical in gonads of members of a population sample taken each month. It will be observed that at all times of the year gonads of some individuals of a population sample may have relatively large amounts of certain constituents, while gonads of other individuals of the same population sample may have a relatively small amount. Certain trends do appear but an average value which emphasizes these trends gives a less true picture of the actual facts than the distribution plot. Statistics calculated from the data are not a truthful representation of the data, because standard deviations and confidence limits are meant to apply to a population

TABLE I

Chemical constituents of gonads of the purple sea urchin (Jan. to Dec. 1956)
(Water in % wet weight, all others in % dry weight)

Date	Av. GI*		Lipid		NPN		Protein		Glycogen		Water		Ash	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
1/25	9.2	7.5	10.0	13.6	1.4	1.2	44.9	31.5	5.9	10.8	65.1	67.0	—	—
2/21	7.6	7.2	20.5	22.0	3.8	3.0	34.2	30.2	5.7	6.6	69.7	71.3	7.1	7.0
3/31	4.4	3.0	12.9	19.4	3.2	2.1	30.7	27.7	3.0	10.2	74.7	77.8	9.5	8.1
4/20	3.5	1.8	19.5	16.1	3.1	3.0	31.1	24.1	14.0	4.1	78.1	76.2	7.4	4.8
5/30	5.9	5.7	14.5	15.4	3.0	2.3	27.0	27.9	10.6	10.3	71.1	58.5	—	2.6
6/17	3.8	4.6	19.8	19.8	2.4	2.0	22.7	23.2	5.2	7.1	70.1	61.3	4.7	2.4
7/31	5.6	10.0	18.5	19.0	2.3	1.8	26.3	26.4	5.7	5.2	68.2	55.0	1.9	2.2
8/30	6.7	6.7	16.3	13.2	2.4	2.6	21.5	18.4	1.0	1.0	63.2	74.3	3.7	2.7
9/27	12.4	15.5	15.5	18.7	2.4	1.8	34.0	26.3	1.6	1.9	68.7	64.9	5.5	3.5
10/31	11.9	12.8	22.4	21.2	2.8	1.5	35.2	35.4	3.4	3.9	70.0	73.5	3.2	2.7
11/28	14.0	14.4	10.5	15.9	2.0	2.0	33.4	35.1	7.8	6.9	69.0	67.0	5.3	4.4
12/18	17.5	16.6	15.9	20.1	3.7	2.3	36.2	39.8	3.2	3.6	66.1	66.3	7.1	5.8
Av.			16.4	24.5	2.7	2.1	31.5	29.0	4.8	6.0	69.5	67.7	4.6	3.8

* GI refers to gonad index obtained as defined in the text. NPN refers to non-protein nitrogen.

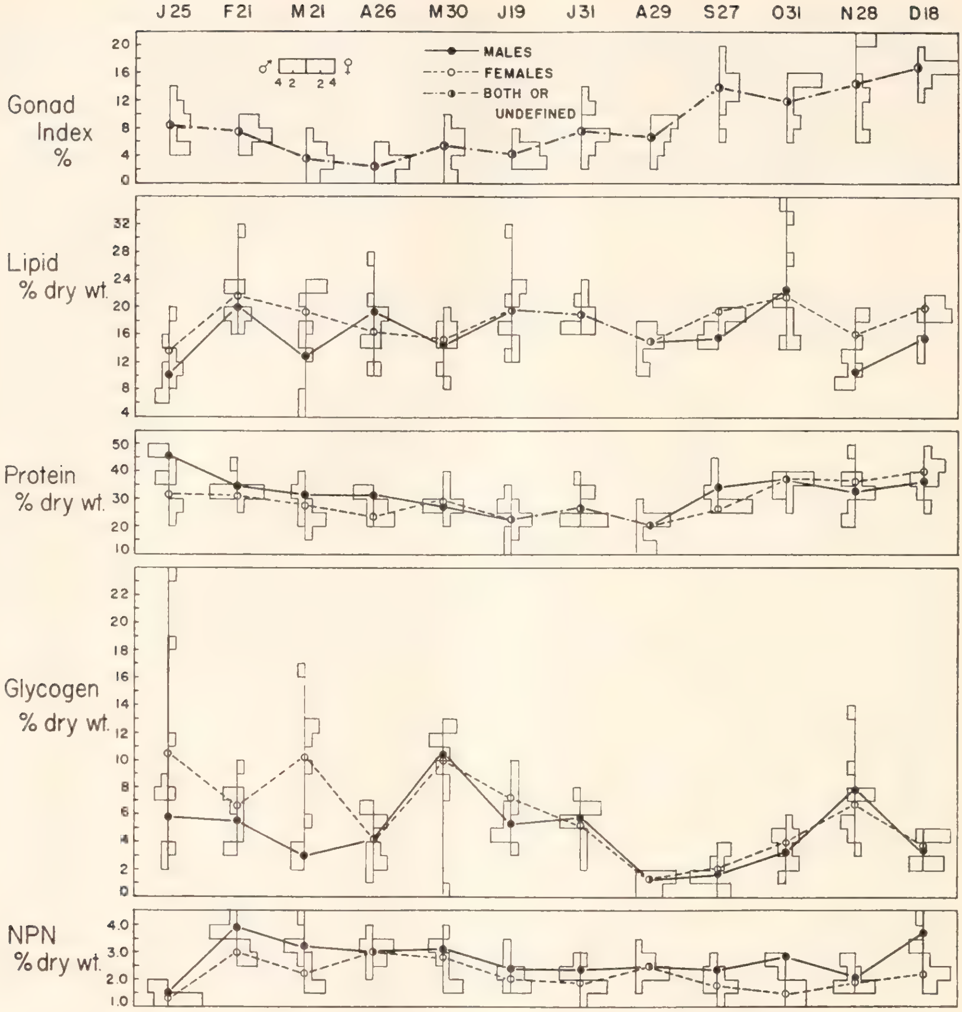

FIGURE 1. The distribution graphs illustrate the inhomogeneity of the population of sea urchins throughout an annual cycle, not only in gonad index but in content of various organic constituents in the gonads (last four graphs). Glycogen content of gonads shows greatest variability, non-protein nitrogen (NPN), least. For explanation see text.

with a normal distribution, not to a skewed one such as is the population dealt with here.

The over-all averages for the entire year disclose some interesting information about the gonads (last line, Table I). The ovary is distinctly richer than the testis in lipid and glycogen but the testis appears to be richer than the ovary in non-protein nitrogen, protein nitrogen and possibly in salts (ash) and water, although the few samples taken and their variability from month to month make any deductions on the latter two substances questionable.

When a sample of animals is selected and the ones with a low gonad index are compared with those with a high index, the contrasts in chemical constitution of gonads during the course of the reproductive cycle are most clearly brought out as seen in Table II. In addition to the chemicals discussed above, it is seen that the RNA per unit weight of the ovary increases with its enlargement while the DNA decreases; in the testis the reverse is true, the RNA per unit weight decreases while the DNA more than doubles.

These differences between ovary and testis are understandable in view of the

TABLE II

Chemical constituents in spent and gravid gonads of the purple sea urchin (in % dry wt.)

Sex and condition	Gonad index	NPN	Protein	RNA	DNA	TN	Reducing sugar
♂ spent	2.3	2.0	23.1	3.25	0.0014	5.7	0.007
	—	2.0	27.4	3.46	0.0010	6.4	
	3.5	2.2	23.7	2.59	0.0018	6.0	
Av.	2.9	2.1	24.7	3.10	0.0014	6.0	
Gravid	17.8	1.3	35.0	5.0	0.0007	6.9	0.037
	18.2	2.1	34.4	4.8	0.00065	7.6	
	18.9	1.0	39.4	3.8	0.00059	7.3	
Av.	18.3	1.5	36.3	4.5	0.00065	7.3	
♂ spent	1.52	4.1	25.9	2.3	4.7	8.3	0.0036
	2.84	2.1	36.0	2.4	4.1	7.9	
	2.43	2.2	36.0	2.1	4.4	8.0	
Av.	2.26	2.8	32.6	2.3	4.4	8.1	
Gravid	21.5	0.66	43.0	1.3	9.8	7.6	0.034
	19.2	1.20	39.8	0.9	8.0	7.6	
	1.77	0.59	42.5	0.9	9.3	7.4	
Av.	19.5	0.82	41.8	1.0	9.0	7.5	
		NPN	Protein	Lipid	Glycogen	TN	Water
♀ spent	1.18	3.75	23.2	9.5	0.41	7.5	63.2
	1.28	3.07	20.1	18.7	3.35	6.3	78.8
	1.49	2.84	28.2	15.5	2.77	7.4	—
Av.	1.32	3.22	23.8	14.6	2.18	7.1	71.0
Gravid	21.8	1.83	33.5	18.1	7.98	7.2	—
	17.0	1.92	26.8	19.9	2.35	6.2	64.9
	17.7	2.41	42.1	21.3	4.54	9.1	70.0
Av.	18.8	2.05	34.1	19.8	4.96	7.5	67.4
♂ spent	1.43	3.03	38.2	17.4	2.63	9.2	—
	3.0	3.14	33.4	14.2	6.41	8.5	76.2
	1.42	3.34	32.4	20.5	1.48	8.5	74.9
Av.	1.95	3.17	34.7	17.4	3.51	8.7	75.5
Gravid	21.3	1.63	37.5	13.0	4.24	7.9	—
	21.0	1.91	24.4	11.0	9.86	5.8	65.1
	21.6	4.47	35.2	12.6	4.43	10.1	66.3
Av.	21.3	2.67	32.4	12.2	6.51	7.9	65.7

gametes produced and their prominence in the gravid gonads. It will be remembered that female sea urchins can usually be distinguished from male sea urchins during all months of the year by the presence of eggs in the ovary, even though the eggs may be small and immature. Only occasional specimens are indeterminate as to sex, either just after spawn-out or because they have not yet matured (very small ones, that is, less than 17 mm. in test diameter are always indeterminate for the latter reason). Conversely, males can usually be detected by the presence of sperm in the testis. Eggs contain considerable stores of food for the development of the embryo while sperm contain only stores for the brief period of locomotion of the sperm preceding fertilization. *A priori,* one expects eggs to be rich in lipids and glycogen, whereas sperm are expected to contain some glycogen as food reserve for movement. One also expects the eggs to contain more RNA than sperm but less DNA. As can be seen from the data, these expectations are indeed realized. More suprising is the fact that the spent or immature gonads also show contrasts in chemical constitution, especially the large lipid content of immature or shrunken ovaries as compared to immature or shrunken testes. Presumably the lipids are present in the ovarian epithelium which gives rise to the eggs. Histochemical studies would be interesting on ovarian and testicular materials at different times in the gonadal cycle.

Table III

Increase in organic constituents of gonads of the purple sea urchin during growth from shrunken to maximal size (in mg.; total wt. in grams)

	♂		Relative increase	♀		Relative increase
	Spent	Gravid		Spent	Gravid	
Gonad index	1.42	21.6	15.2 ×	1.18	21.8	18.5 ×
Total wt. (arbitrary) in grams	1.0	15.2	15.2 ×	1.0	18.5	18.5 ×
Total nitrogen, mg.	84	1170	14.0 ×	65.5	1369	21.0 ×
Non-protein nitrogen, mg.	29.9	266	8.9 ×	26.6	328	12.3 ×
Protein, mg.	336.5	5639	16.7 ×	243	6512	26.7 ×
Lipid, mg.	174	1854	10.4 ×	146	3663	25.1 ×
Glycogen, mg.	35	989	28.2 ×	21.8	917.6	42.1 ×
Reducing sugar, mg.	0.036	5.16	143.0 ×	0.07	6.84	98.0 ×
RNA, mg.	23	152	6.6 ×	31.0	832.5	26.8 ×
DNA, mg.	44	1368	31.1 ×	0.014	0.120	8.6 ×

It is not possible to ascertain productivity of organic materials in the gonads of the sea urchin on a per unit weight basis, because all that is then observed is a shift in emphasis on certain materials, which accompanies the onset of maturity, *i.e.,* a synthesis of some materials at a greater rate than that of others. Furthermore, the relative content of water in the ovary declines to some extent concomitantly with a general increase in the total mass of other substances in the ovary. Therefore, to ascertain organic productivity of the gonads it is necessary to take into consideration the increase in mass of the gonads, as well as their change in chemical constitution (per unit weight) during the growth from a spent to a fully gravid condition. Gonads increase in mass by a ratio which equals the gonad index of a gravid animal divided by the gonad index of a spent animal. For a female this is 18.5-fold, for a male it is 15.2-fold (using the data for maximal and minimal sizes of gonads given in Table II). If the gonads of a spent animal weigh 1 gram, as they would in fact for an average-sized animal of 90 grams total wet weight, then the ovaries of a gravid female of this size would weigh 18.5 grams and the testes of a gravid male of this size would weigh 15.2 grams. The content of each chemical constituent in the spent and gravid gonads of animals of this size could then be calculated by multiplying the weight of the gonad in grams by its per cent content of each of the constituents given in Table II. Data so calculated are given in Table III. By dividing the content of each constituent in the gonad of a gravid individual by the content of that constituent in the gonad of a spent animal, the relative increase in mass of the chemical constituent in question during the growth of the gonads from the spent to the gravid state was calculated and the data are given in Table III. For example, to obtain the content in NPN in a spent ovary its weight, 1000 mg., is multiplied by the average fractional content[4] of NPN in spent ovaries, 2.66 per cent or 0.0266, giving 26.6 mg. To calculate the NPN in a gravid ovary its weight, 18,500 mg., is multiplied by the average fractional content of NPN in gravid ovaries —1.775 per cent or 0.01775. This gives a value of 328 mg. The increase in mass of NPN from spent to gravid condition is then 328 divided by 26.6, which is 12.3 times.

The chemical constituents showing the most striking total increases during the growth of the gonad observed in Table III are of course the ones which have also increased on a per unit weight basis. It will be seen that the total amount of DNA in the testis increases by about 31 ×, the RNA in the ovary 27 ×, the glycogen in the testis 28 ×, the glycogen in the ovary 42 ×, the lipid in the ovary 25 ×, the lipid in the testis 10 ×, the protein in the testis 17 ×, the reducing sugar in the testis 143 × and in the ovary 98 ×.

DISCUSSION

It is interesting at this time to inquire about several matters concerning the gonadal biochemical cycle in the purple sea urchin. To what extent is it possible to explain the chemical diversity in gonads in a population of sea urchins selected at random at any time during the year? How does the build-up of the nutrients in the gonads occur? What is the over-all productivity of the purple sea urchin?

The variability of chemical constitution of the gonads of the sea urchin during the year may be just another index of the failure to get synchronized spawning in this species. At almost all times the population is rather inhomogeneous with respect to the gonad cycle, some animals having fairly well-developed gonads while others are poorly developed or spent. Only in March and April is the gonad index rather low for most specimens and only in December is it consistently high. Biochemical inhomogeneity of different individuals may therefore reflect population inhomogeneity in gonadal development. Even when animals of like gonad index are compared, however, one finds biochemical differences. Perhaps an individual just spending or one just building up to the same intermediate gonad index, may be quite different histologically and histochemically. Information on this as a possible explanation of chemical inhomogeneity is lacking at the present time.[5]

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[4] The average of the values for the two groups of spent animals in Table II, namely, 2.1 and 3.2 per cent, giving 2.65 per cent or 0.0265.

Another factor which may play a role in the variability in chemical constitution of the sea urchin gonad is availability of nutrients at different times during the year, or at any one time, a difference in availability of nutrients to each individual in the population. The relative immobility of the urchins which have bored their way into the soft rocks makes them dependent upon what grows in their immediate vicinity or what the waves may bring to them by chance. The gonad is the main storage organ of the sea urchin, a little organic material also being stored in the gut (Hilts and Giese, 1949). When an urchin is starved the gonad shrinks and its gonad size may decline even without spawning. However, the intestines of almost all urchins from the field are filled with algae; therefore food seems to be generally available. The purple sea urchin's willingness to eat almost any food, animal or plant, when starved, makes it seem unlikely that it lacks in quantity of food in nature. However, the food may have unequal nutritive quality at different times. No evidence was collected upon this point, but young growing algae are known to contain much protein while old ones are made up, to a considerable extent, of polysaccharides which are probably a much less available source of food (Wort, 1955). The availability of nutrients may therefore vary even though the bulk of food taken in may be the same.

The build-up of nutrients in the gonads must be a relatively slow process, yet the increase in organic matter during a gonadal cycle is rather striking, indicating effective digestion, mobilization, and conversion of food. Digestion appears to be a rather slow process in the sea urchin, since algae may be defecated for several weeks from a single gutfull in an animal deprived of further sources of food. While the enzymes of the sea urchin readily handle proteins and starch, they attack few of the polysaccharides of the algae (Lasker and Giese, 1954; Huang and Giese, 1958). However, bacteria may play a role in digestion since they readily hydrolyze the algal polysaccharides in the gut of the urchin. Where the nutrients go when they leave the intestine is not clear. The perivisceral fluid contains some protein, reducing sugar, lipid and very little non-protein nitrogen. Most of the protein forms striking fibrous clots. When these are filtered out the remaining fluid appears to be protein-free (TCA negative).[6] It is possible that the continual dribble of sugar, amino acids, and possibly lipids, from the intestine into the body fluid, is adequate for the build-up of the reserves in the gonads. However, it is desirable that someone explore other pathways of nutrient transport, particularly by wandering amebocytes and by the haemal system which extensively vascularizes both the gut and the gonads (Hyman, 1955).

To assess the over-all productivity of the sea urchin it is necessary to consider not only the gonad cycle and the increase in organic material which occurs there, but also other possible constituents which accumulate organic materials. The only organ of considerable size in the sea urchin other than the gonad is the intestine, but some tissue is also present in the water vascular system, the muscles of the spines and pedicellariae, the dermal branchiae, the epidermis, the mesenteries, and the coelomic lining. In an urchin of about 90 grams, all of these structures are estimated to weigh about 7 grams.

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[5] That the small size of the sample of the population is not the cause of the variability of the gonads is shown by a study with larger sample sizes by Josef Miller of Monterey Peninsula College. He compared the gonad index of samples of 10, 20, 40 and 80 sea urchins. The gonad index for a given population of sea urchins at a given season was almost the same, within a few per cent, regardless of the sample size.

[6] However, two protein peaks are disclosed in paper electrophoresis studies of fluid filtered after clotting (Favour and Giese, unpublished).

If, for purposes of argument this figure is tentatively accepted, then the total increase in organic material with one gonadal cycle is approximately three-fold. Unfortunately we do not know how many gonadal cycles a single sea urchin can undergo in one season. The fact that a population of sea urchins collected at almost any time of the year, with the exception of the time of the highest gonad index and the period just after the maximal spawn, shows individuals with widely different indices (see Figure 1 and the figures in Bennett and Giese, 1955), suggests that a single individual may spawn several times during the year. If this is true, several times the above figure may be a more nearly correct estimate of production of organic material. Since the sea urchin also grows in diameter and bulk, the true figure must be larger on this account as well. We do not at present have sufficient data to make a determination of the growth rate and the rate of incorporation of nutrients into body material.

SUMMARY

1. Monthly determinations were made of the amount of lipid, glycogen, non-protein nitrogen, protein, water, and ash present per unit weight in gonads of the purple sea urchin, *Strongylocentrotus purpuratus.* Tests for reducing sugar, DNA and RNA were made for gonads at the height of the reproductive season and after spawning-out.

2. A change in relative proportions of the chemical constituents was observed with maturation of the gonads. In the ovary protein, lipid, glycogen, reducing sugar and RNA increase proportionally more than the over-all increase in bulk of the gonad, while DNA and possibly water, increase proportionally less. In the testis, glycogen, reducing sugar, DNA and possibly protein, increase proportionally more than the over-all increase in bulk, while RNA, lipid, and possibly water, increase less than the increase in total bulk.

3. A considerable increase in the total amount of all the organic constituents tested here occurs during the growth of gonads. Thus, a gravid ovary is about 18.5 times the bulk of a spent one and a gravid testis is about 15.2 times the bulk of a spent one.

4. The sources of nutrients and the possible transport are discussed with reference to the literature.

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THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. I. A METHOD FOR MEASURING THE RATE OF CALCIUM DEPOSITION BY CORALS UNDER DIFFERENT CONDITIONS

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The purpose of this study is to examine the rate of growth of reef-building corals by measuring the calcium deposition in the skeleton with the aid of a new method using radioactive calcium-45 as tracer. With this procedure it was possible to determine calcification rates in the different parts of coral colonies, and to estimate quantitatively the effect of light and darkness, zooxanthellae and carbonic anhydrase inhibitors on skeletogenesis.

Numerous attempts have been made in the past to estimate the growth rates of reef-building corals, mostly by letting weighed and measured coral colonies grow in their natural habitat for periods of months to years (Agassiz, 1890; Abe, 1940; Boschma, 1936; Edmondson, 1929; Kawaguti, 1941; Ma, 1937; Mayor, 1924; Motoda, 1940; Stephenson and Stephenson, 1933; Tamura and Hada, 1932; Vaughan, 1919). Recently, Kawaguti and Sakumoto (1948) tried, by a chemical method, to determine the rate of calcium uptake of corals in light and darkness.

Using calcium-45 as tracer, we have developed a rapid and precise method for measuring the rate of incorporation of calcium into the coral skeleton under controlled laboratory conditions (Goreau, 1957). The preliminary experiments, described here, were carried out on the following coral species: *Manicina areolata* (Linné), *Cladocora arbuscula* (Lesueur), *Porites divaricata* (Lesueur), *Acropora prolifera* (Lamarck), *Madracis decactis* (Lyman) and *Oculina diffusa* (Lamarck) from Jamaica, B. W. I.; *Acropora conferta* (Quelch) from Eniwetok Atoll; and *Montipora verrucosa* (Lamarck), *Porites compressa* (Quelch), *Pocillopora damicornis* (Linné) and *Porolithon* sp., a coralline alga, from Hawaii.

All the madreporarian corals used in these experiments are shallow-water forms which contain zooxanthellae. Among these, *Oculina diffusa* is the only species which has not been collected from reefs, but it is common in Kingston Harbour where it grows on rocks on a muddy bottom (Goreau, 1958). The Hawaiian *Porolithon* listed above is a calcareous alga of the family *Corallinaceae*, representatives of which are important reef builders in the Central Pacific (Emery, Tracey and Ladd, 1954).

PROCEDURE

Freshly collected coral colonies in good condition were put into glass vessels containing filtered sea water and fitted with tight covers. Aeration, circulation and pH were maintained by bubbling a slow stream of air through the water. The temperature was kept to within 1° C. during the experiments (about 25° C. in Jamaica and Hawaii, 28.5° C. in Eniwetok) by keeping the vessels partly immersed in a water bath. After allowing the coral to acclimatize for twenty-four hours, neutralized $Ca^{45}Cl_2$ was added to give about 20,000 c.p.m./ml. of sea water. The amount of calcium thus added was less than five per cent of the total dissolved Ca^{++} already present. The initial activity was determined by counting 60-μl aliquots taken from each vessel after one hour, to allow for complete mixing of the isotope.

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In addition to the living corals, pieces of clean dead corallum from the same species were included in each vessel to act as controls for measuring the inorganic isotopic exchange rate of the coral skeleton during the experiments.

Samples of coral and water were repeatedly taken, starting with three hours from the beginning of the experiment, by the following method: a coral colony, together with its control, was removed from the vessel and small pieces were cut off with scissors or cutting pliers. From five to fifteen replicate samples of about one hundred milligrams each were taken at a time. Samples were collected only from homologous parts of the colonies. This was particularly important in branching corals such as *Acropora* and *Porites* where there were shown to be strong differences in the rate of calcium uptake between the apical and lateral branch polyps.

The coral pieces were placed on filter paper to remove excess radioactive sea water, then washed in five two-minute changes of slightly alkaline distilled water. After this, each sample was dissolved in a separate tube containing two milliliters dilute HCl, and heated to boiling. The coral suspension was homogenized to disperse the organic matter. The contents of each tube were made up to five milliliters with distilled water, and a 500-μl aliquot was taken for Kjeldahl nitrogen determination.

The calcium in each tube was precipitated as the oxalate by the method of Vogel (1943), and filtered out on pre-weighed Whatman No. 42 filter paper planchets, using a cone to spread the precipitate in circles of uniform diameter. The dried and weighed samples were counted with an end window G-M tube, and the observed activity corrected for self-absorption.

In the early stages of these investigations, the question arose of choosing a suitable parameter on the basis of which the calcium uptake could be expressed. For example, Mayor (1924) measured coral respiration in terms of tissue weight after the corallum had been dissolved with nitric acid; Odum and Odum (1955) determined biomass by loss on ignition at 600° C.; and Kawaguti and Sakumoto (1948) measured calcification rates per gram coral. None of these methods was considered satisfactory. The writer had previously used organic nitrogen as a measure of total cellular matter in corals (Goreau, 1956). The relationship of organic nitrogen to tissue weight was determined for the polyps of *Mussa angulosa*, a coral from which fairly large skeleton-free pieces of tissue could be readily obtained. In this species nitrogen constituted 2 per cent of the wet weight and 11.2 per cent of the dry weight. All results, save those of the exchange controls which lacked tissue, were expressed in terms of calcium deposited per milligram of nitrogen, on the assumption that the nitrogen is a measure of the total coral (plus zooxanthellae) protein present. Nitrogen was determined by the micro-Kjeldahl method of Ma and Zuazaga (1942).

The amount of calcium taken up by the coral was calculated from the specific activity of the sea water in the vessels. This was determined by counting 60-μl water aliquots spread to a constant diameter in lens paper circles mounted on microscope coverslips and dried under a lamp. The observed count was corrected for self-absorption and the specific activity of the water calculated from its calcium content.

The Calcium Exchange in the Skeleton Controls

Equilibrium exchanges of calcium between the skeleton and sea water were determined on samples of dead coral devoid of tissue, and run at the same time as the living experimental colonies. Isotopic equilibrium appeared to be established

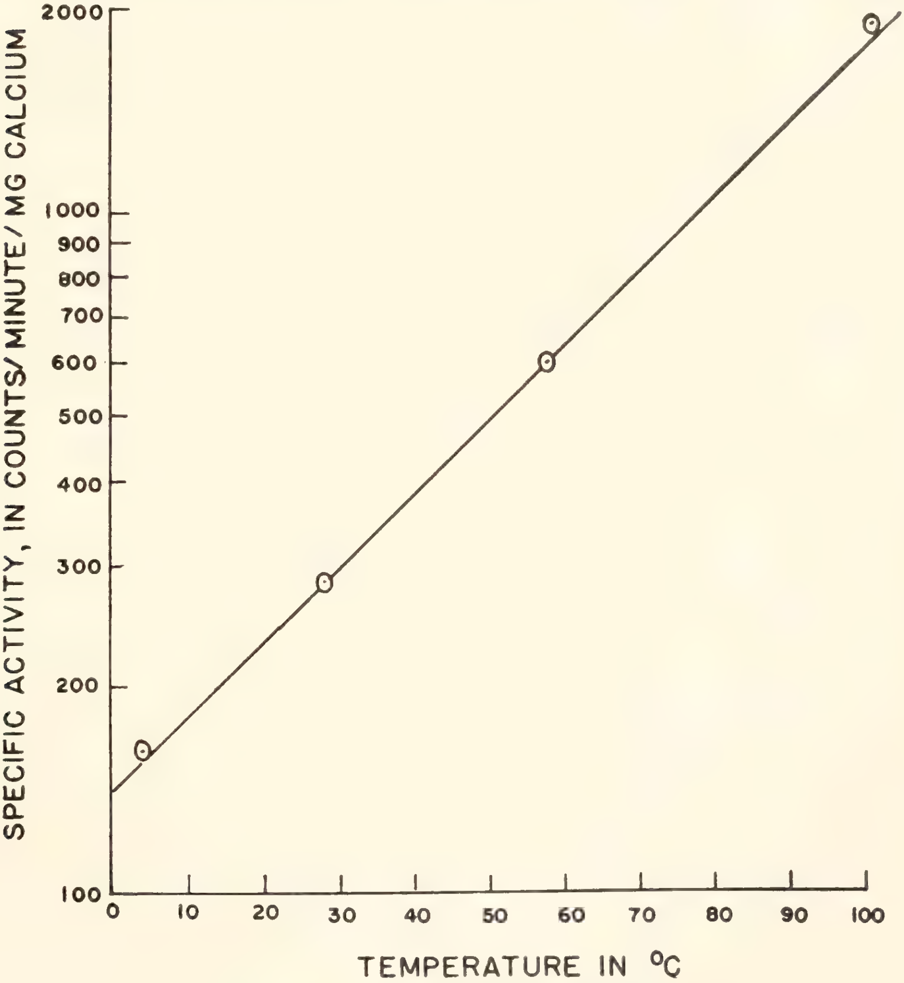

Figure 1. Calcium-45 exchange of small pieces of corallum from *Manicina areolata* with sea water at 4° C., 28° C., 58° C. and 100° C. The coral was carefully cleaned to remove all organic matter, and the experiments ran for twenty-four hours. The ordinate is the specific activity plotted on a logarithmic scale.

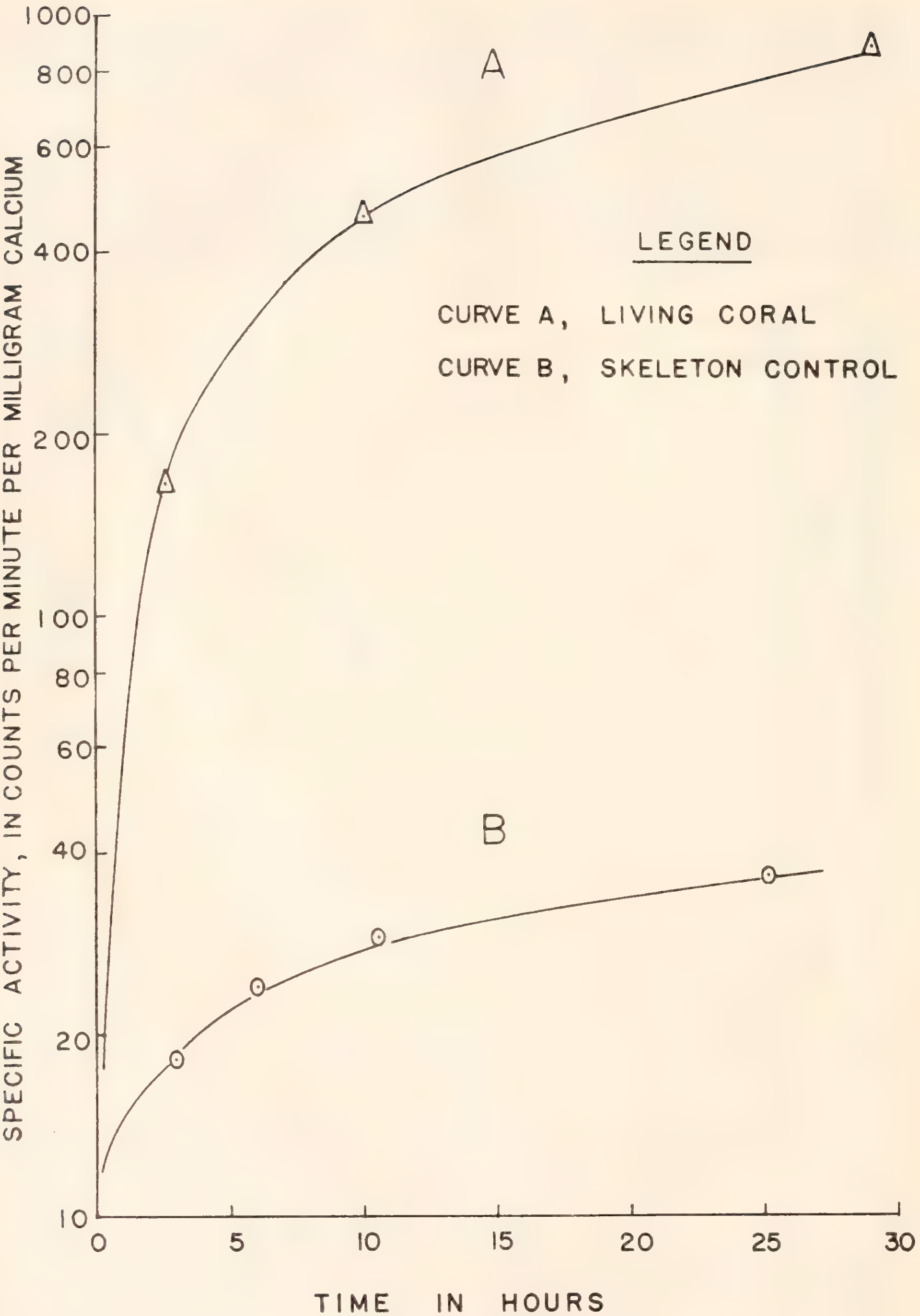
1000
800
600
400
200
100
80
60
40
20
10
SPECIFIC ACTIVITY, IN COUNTS PER MINUTE PER MILLIGRAM CALCIUM
A
B
LEGEND
CURVE A, LIVING CORAL
CURVE B, SKELETON CONTROL
0
5
10
15
20
25
30
TIME IN HOURS

FIGURE 2.

rather slowly, but in most species tested, the process was sixty to eighty per cent complete at the end of twenty-four hours. As expected, the rate of exchange with sea water was strongly temperature-dependent. This is demonstrated in Figure 1, which shows the specific activity of small pieces of *Manicina areolata* which have been allowed to equilibrate at different temperatures in sea water containing calcium-45. In most species tested, the rate of calcium-45 deposition in the living coral was much faster than in the skeleton controls. This is shown in Figure 2 for *Acropora prolifera,* in which the specific activity of the dead corallum is about five per cent that of the living coral at the end of twenty-nine hours. In water of a given specific activity the equilibration rate appears to be much slower in imperforate corals such as *Oculina* or *Phyllangia* than in perforate species such as *Acropora* or *Porites.* The effect of the total skeletal surface on the exchange rate is being studied.

There is some evidence that the living coenosarc forms a barrier which restricts calcium exchange of the skeleton with the sea water. In a number of experiments in which the calcium rate of the experimental colonies was very low, it was noted that the specific activity of the skeleton controls was higher than that of the living coral. It has been previously demonstrated by Goreau and Bowen (1955) that the exchangeable calcium in the tissues of the cold water coral *Astrangia danae* is maintained at only about eighty-eight per cent of the calcium concentration in the sea, *i.e.,* calcium tends to be excluded from the tissues of coral. Until more evidence is available, it is difficult to state precisely the extent to which coral tissues can restrict the calcium exchange of the underlying skeleton with sea water. This problem is now under investigation.

The Effect of Light on Calcium Deposition in Corals and Other Hermatypes

Light has long been recognized as an essential environmental factor in the growth of tropical reef building corals (Vaughan, 1919; Edmondson, 1928; Verwey, 1930; Kawaguti, 1937a, 1937b; Yonge, 1940; Vaughan and Wells, 1943) and other hermatypes such as *Lithothamnion* and *Millepora.* Yonge and Nicholls (1931a), Yonge (1940) and Kawaguti (1944) stated that this was due to photosynthesis by unicellular zooxanthellae contained within the cells of the gastrodermis. Kawaguti and Sakumoto (1948) claimed that in five species of reef corals the uptake of calcium was greater in light than in darkness. Their observations were based on changes in the calcium content of small volumes of sea water when corals were put in, the results being expressed in terms of milligrams of calcium taken up per hour per gram of coral.

In our experiments, the effect of illumination on deposition of calcium-45 was determined by exposing one series of coral colonies to a standard light source while keeping a control series in darkness under otherwise equal conditions. The light source was a twin bank of 20-watt fluorescent tubes in a reflector housing located about one foot above the experimental vessels.

FIGURE 2. Comparison of the calcium-45 deposition and exchange in living and dead colonies of *Acropora prolifera.* The results from the living coral have been re-calculated in terms of the specific activity to permit direct comparison with the exchange controls which were devoid of organic matter. Both controls and experimentals were run under identical conditions at the same time. The specific activity is plotted on a logarithmic ordinate.

The results of our preliminary experiments are given in Table I which shows calcium uptake in nine species of coral, and a coralline alga (*Porolithon*). In two of these species, dark experiments were not run; only the results of light experiments are shown. In most species, there was a significant increase in the calcification rate on exposure of the coral to a light. The course of a typical experiment is seen in Figure 3 which shows the progressive incorporation of calcium-45 into the skeleton of the Caribbean staghorn coral *Acropora prolifera* in light and darkness.

The pH in both light and dark vessels was measured every six hours with a Beckman Model G pH meter. This showed that the observed differences in the calcification rate in the light and dark experiments were not due to a decrease in the pH of the water of the dark experiments, as such changes were prevented by continuous aeration with a stream of air. It is probable that the negative calcium balance found by Kawaguti and Sakumoto (1948) in some corals in darkness was caused by a lowering of the pH, due to the failure of these workers to aerate or stir the water in their experimental vessels.

TABLE I

Calcification rates in the apical polyps of branching coral species, in μg calcium mg. N^{-1} hr.$^{-1}$
Number of samples in brackets

Species	Calcification in light	Calcification in dark	P
Cladocora arbuscula	6.3±1.58 (9)	6.1±0.20* (10)	0.7
Porites divaricata	9.8±0.54 (10)	5.0±1.00 (8)	0.01
Porites compressa	7.8±1.70 (11)	7.4±2.10 (7)	<0.7
Acropora prolifera	12.4±6.50 (12)	7.2±5.00* (11)	<0.05
Acropora conferta	8.2±3.76 (10)*	— —	—
Montipora verrucosa	11.9±5.60 (9)	9.7±3.4 (10)	0.3
Pocillopora damicornis	10.3±3.90 (11)	6.8±2.1 (10)	<0.03
Madracis decactis	1.0±0.49 (12)	— —	—
Oculina diffusa	1.6±0.38 (7)	0.8±0.15* (9)	0.01
Porolithon sp.	8.8±0.58 (11)	3.3±0.55 (13)	<0.001

* Measurements made on individual polyps.

As seen by the standard deviations of the results, there were usually large variations in the calcium uptake rates of individual samples even if these were taken from adjacent morphologically comparable regions of the same colony. This was never true of the exchange controls. The scatter was not attributable to injury, as all damaged corals were discarded, and the error in counting, weighing and nitrogen determinations was kept below three per cent. In regard to this, our tentative interpretation is that the calcification rates of individual polyps fluctuate, and that some are in a resting stage while others are more or less vigorously growing.

THE EFFECT OF THE REMOVAL OF ZOOXANTHELLAE ON THE CALCIFICATION RATE OF SOME REEF CORALS

All tropical reef-building corals contain zooxanthellae. Their presence as intracellular symbionts in the tissue of the coelenterate host has resulted in a great deal of controversy as to their possible role in the biological economy of the coral reef and its component animals. Boschma (1924, 1925a, 1925b, 1925c, 1926, 1929)

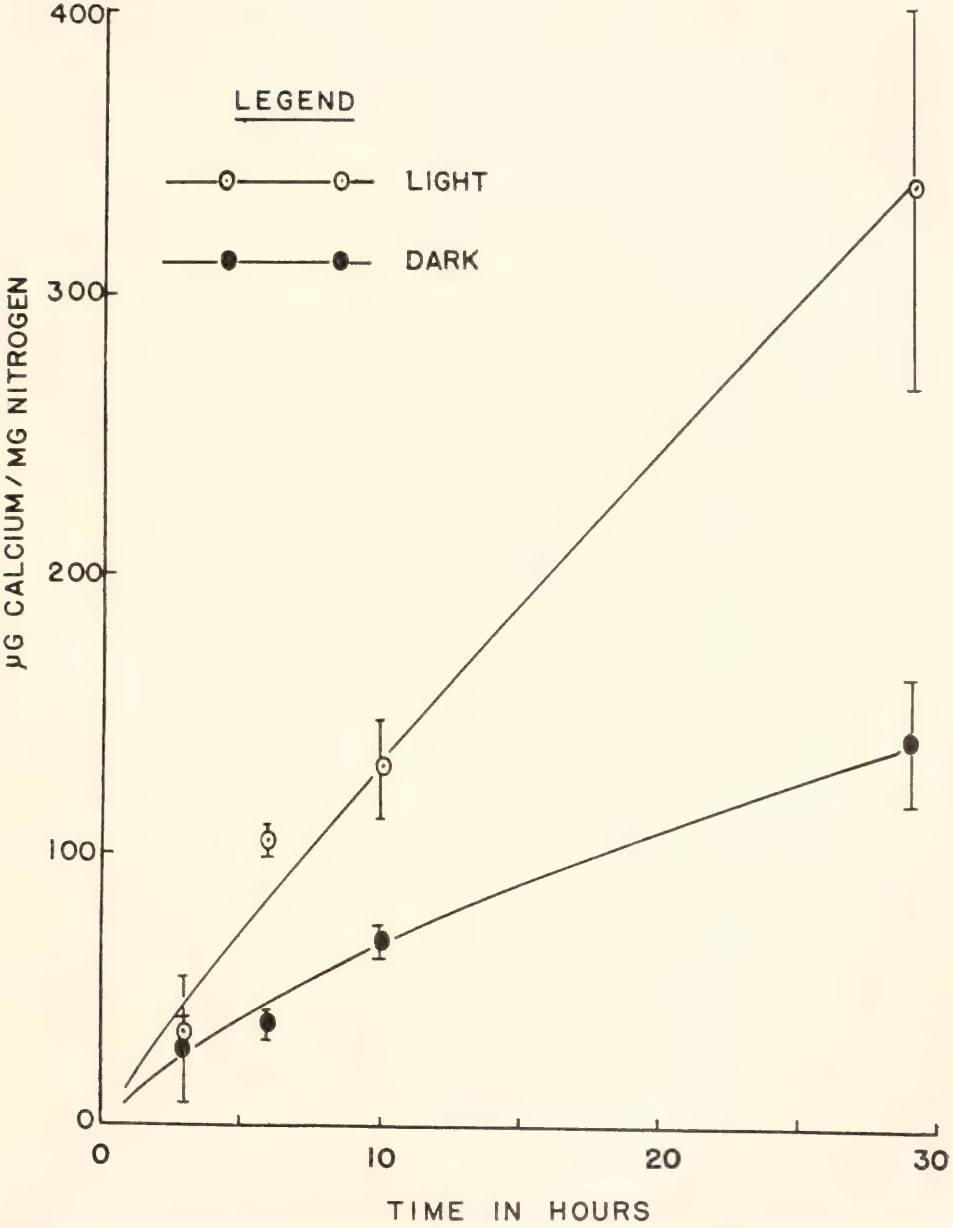

FIGURE 3. The progressive incorporation of calcium into the skeleton of *Acropora prolifera* in light and dark. The results are expressed as µg calcium taken up per milligram nitrogen. The vertical lines drawn through the points represent the standard deviation of the means.

concluded that corals could digest zooxanthellae in the lateral lobes of the mesenterial filaments when no animal food was available. Yonge and Nicholls (1930, 1931a and 1931b) demonstrated that, under the conditions of their experiments, corals were unable to derive enough food from the zooxanthellae to prevent starvation if deprived of their normal animal food supply. They also showed that zooxanthellae could not be digested by corals due to the absence of carbohydrate-splitting digestive enzymes and that these algae were extruded intact and in large numbers when the coral was kept in darkness for long periods of time, or whenever the metabolic rate of the coral was depressed, *i.e.,* by starvation or high temperature. The question of whether or not the reef-building corals are at least in part herbivorous, *i.e.,* feeding on their zooxanthellae, has recently been revived by Sargent and Austin (1954) and Odum and Odum (1955) who concluded from their productivity studies that at least some of the organic matter produced by zooxanthellae and boring algae may be utilized by the coral host. Unfortunately, these authors were unable to verify the existence of such an internal food cycle by experimental means. At the present time, it is still necessary to agree with the conclusions of Yonge and Nicholls (1930, 1931b) that reef corals are specialized carnivores, the exceptional proliferative powers of which are probably due to an increased metabolic efficiency made possible by the ability of the zooxanthellae to assimilate many of the metabolic waste products of the animal host.

TABLE II

Calcium uptake by colonies of Oculina diffusa and Manicina areolata
in presence and absence of zooxanthellae
Number of samples in brackets

Species	Light		Dark	
	With zooxanthellae	Without zooxanthellae	With zooxanthellae	Without zooxanthellae
*O. diffusa**	1.63± 0.38 (7)	0.37±0.01 (6)	0.81± 0.15 (9)	0.26±0.01 (5)
*M. areolata***	462.00±63.20 (11)	28.40±7.80 (9)	71.70±14.90 (8)	30.20±6.20 (10)

* Measurements made on individual polyps, in μg Ca mg. N^{-1} hr.$^{-1}$.

** Individual samples taken from different colonies, calcium uptake expressed in counts per minute per milligram skeletal calcium at eighty hours.

The zooxanthellae *per se* are not necessary to individual coral polyps, nor do they appear to be directly linked with the calcification process since they are absent from deep sea and cold water corals, while they are present in many non-calcareous tropical shallow water coelenterates.

We have determined the effect of the presence or absence of the zooxanthellae on reef coral calcification in *Manicina areolata* and *Oculina diffusa.* Colonies of these corals, which are normally yellowish or greenish brown in colour, were kept in circulating sea water in darkened tanks for periods of about six weeks, to cause gradual extrusion of the zooxanthellae. The experiments were run only when the coenosarc of the corals became completely colourless and transparent, and when small pieces failed to give the chlorophyll test on extraction with eighty per cent

TABLE III

Calcification rates in different parts of branching coral colonies, in μg Ca mg. N^{-1} *hr.*$^{-1}$
Number of samples in brackets

Species	Apical polyps of primary branches	Lateral polyps
M. verrucosa	11.8±3.90 (9)	1.38±0.50 (6)*
P. compressa	7.8±1.70 (11)	1.55±0.20 (5)*
P. damicornis	6.8±2.65 (11)	1.31±0.72 (6)*
A. conferta	8.2±3.76 (10)	1.87±0.92 (8)**

* Lateral polyps taken from base of branch.
** Apical polyps of secondary branches.

acetone. These decolorized corals were at all times fully expanded and appeared to be normal, except for the lack of zooxanthellae.

The experiments were conducted in both light and darkness, as described in the foregoing section. In the two species observed so far, loss of the zooxanthellae caused the rate of calcium deposition to fall to very low levels as shown in Table II. The results for *Manicina areolata* are expressed in terms of the specific activity owing to the accidental loss of the nitrogen samples. The experiment on *Oculina diffusa* ran for eight days and the results are given in terms of the nitrogen content. It is significant that removal of the zooxanthellae almost abolishes the response of the calcification reaction to light which is seen in the normal controls containing zooxanthellae.

Although the zooxanthellae seem to play an important role in determining calcification rates in reef-building corals, certain, as yet unknown, physiological factors operate to control the basic mineralization process in a manner which bears no obvious relationship to the number of algae present in a given species. This is illustrated by the fact that large apical polyps of some of the branching acroporid corals contain few zooxanthellae but calcify several times faster per unit of tissue nitrogen than the yellowish brown lateral polyps which are literally stuffed with algae.

TABLE IV

Calcium-45 uptake of coral treated with 10^{-3} *M Diamox*
in light and darkness, in μg Ca mg. N^{-1} *hr.*$^{-1}$
Number of samples in brackets

Species	Light control	Light with Diamox	Dark control	Dark with Diamox
M. decactis	0.98±0.49 (12)	0.56±0.05 (10)		
P. divaricata	9.80±0.54 (10)	4.80±0.55 (8)	5.00±1.00 (8)	3.3±0.20 (11)
C. arbuscula	6.30±1.58 (9)	3.40±0.95 (10)	6.10±0.20 (10)	3.6±0.55 (7)
O. diffusa				
Zooxanthellae	1.63±0.38 (7)	0.30±0.02 (8)	0.81±0.15 (9)	Not measurable (6)
No zooxanthellae	0.37±0.01 (6)	Not measurable (6)	0.26±0.01 (5)	Not measurable (6)

CALCIFICATION RATES IN DIFFERENT PARTS OF A CORAL COLONY

A glance at any living coral will show that there must be large variations in the growth rates of different parts of the same colony, especially in branching species. A field analysis of the differential growth pattern of reef corals was published by Stephenson and Stephenson (1933). With our method, the growth rates in different parts of the same colony were quantitatively measured. Studies on four species of branching corals, summarized in Table III, show that the calcification rates of the apical parts of such corals are from four to eight times faster than growth in the lateral and basal regions. Well developed calcification gradients are found in corals which have a strongly oriented growth pattern. An example of this is seen in the important Pacific reef-building coral *Acropora conferta* in which the primary direction of growth is horizontally outward from a center, resulting in the formation of large tabular colonies. The main growth occurs in the tips of numerous radially outgrowing branches, the apical polyps of which are colored a pale pastel mauve. The apical polyps of the secondary branches are still pale but smaller, whereas those of the tertiary branches are almost indistinguishable from the yellowish brown lateral polyps. The results of a typical experiment are summarized in Figure 4, the location of the different branches being shown in the diagram.

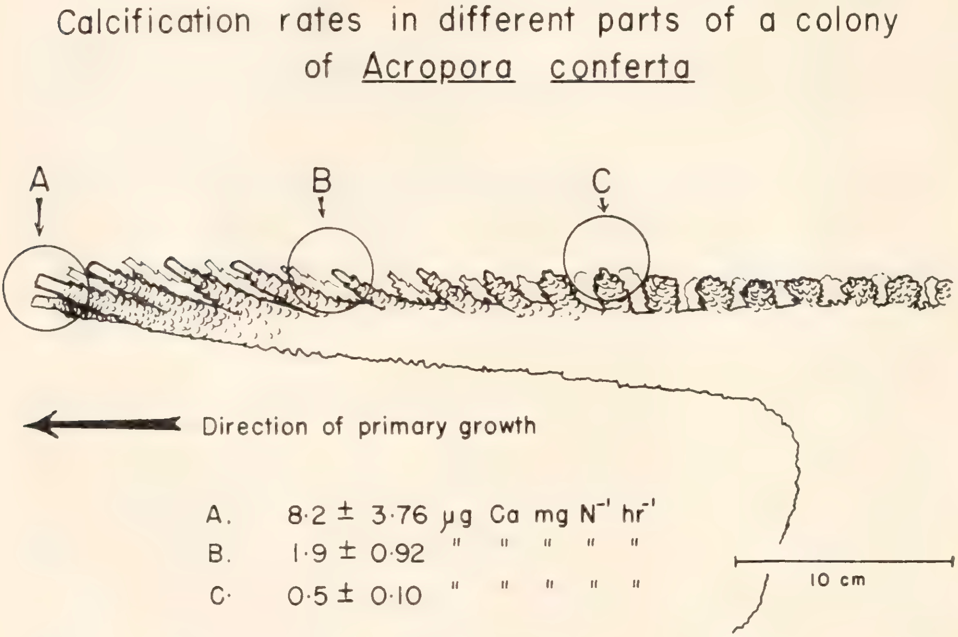
Calcification rates in different parts of a colony
of Acropora conferta
A
B
C
Direction of primary growth
A. 8·2 ± 3·76 µg Ca mg N⁻¹ hr⁻¹
B. 1·9 ± 0·92 " " " " "
C· 0·5 ± 0·10 " " " " "
10 cm

FIGURE 4. Calcification rates in three different parts of a colony of *Acropora conferta*. Only the apical polyps were sampled, their relative positions being indicated by the circles on the diagram. The calcium deposition rate is highest in the large pale apical polyps which are oriented in the direction of primary growth, and marked by circle A. At positions B and C, progressively further away from the growing edge of the colony, the calcification rate becomes greatly reduced.

The Effect of a Carbonic Anhydrase Inhibitor on Calcium Deposition in Corals

Wilbur and Jodrey (1955) demonstrated that shell formation in the oyster *Crassostrea virginica* was greatly reduced in the presence of small concentrations of certain heterocyclic sulfonamides which are powerful specific inhibitors of the enzyme anhydrase. In a series of unpublished experiments we found this enzyme present in all of the twenty-three coral species that were tested. Although carbonic anhydrase was also found in several species of sea anemones and zoanthidea, none of which are calcareous, it was of some interest to determine whether the inhibition of this enzyme had any effect on the calcification rates of corals. The inhibitor used in these preliminary experiments was 2 acetyl-amino 1,3,4,diathiazole-5-sulfonamide, or Diamox. This compound was supplied through the kindness of the Lederle Laboratories Division of the American Cyanamid Division. The experiments were carried out by placing healthy coral colonies into a 10^{-3} *M* (approx. 1:20,000) solution of Diamox in sea water and adding calcium-45 twelve hours later. The experiments were run in light and dark, each having a control without Diamox. All corals used in these experiments could survive 1:20,000 Diamox for at least two weeks, provided they were kept in the light. In darkness, survival time was reduced to about five or six days.

In *Porites divaricata*, a fast growing shallow-water coral that tolerates strong light, treatment with 10^{-3} *M* Diamox in the light caused a fifty-one per cent fall in the calcification rate. Exclusion of light caused the calcium uptake to fall a further thirty-four per cent in the presence of Diamox, as shown in Table IV. It is interesting to note that, in this species, the inhibitor had about the same effect as exclusion of light, both causing a fall of about fifty per cent in the calcification rate. This seems to indicate that, as far as their potentiating effect on the calcification rate is concerned, the action of carbonic anhydrase and that of photosynthesizing zooxanthellae are similar and probably synergistic.

In *Cladocora arbuscula*, a coral which grows best in a somewhat deeper and shadier environment, exclusion of light appears to have relatively little effect on the calcification rate, as shown in Table IV, and the per cent inhibition of the calcium uptake produced by Diamox is about the same in light as in darkness. Thus, in this coral, the zooxanthellae appear to play a much less important part in the calcification process than carbonic anhydrase.

In *Oculina diffusa,* the relative effects of carbonic anhydrase and zooxanthellae could be studied in more detail since it was possible to grow this coral without its algae. In the presence of zooxanthellae, there was a fifty-nine per cent decrease in calcium uptake on exclusion of light, whereas Diamox in the light caused approximately eighty per cent inhibition. In darkness, with zooxanthellae and in the presence of Diamox, calcification could not be measured under the conditions of our experiment. Similar results were found in light and darkness in zooxanthellae-less colonies, where there appeared to be practically complete cessation of measurable calcification in the presence of Diamox. These results indicate that in this species carbonic anhydrase exerts a somewhat greater effect on the calcification rate than do the zooxanthellae.

In all four species of reef corals so far tested, 10^{-3} *M* Diamox caused a forty per cent to fifty per cent decrease of the calcification rate. This concentration of Diamox

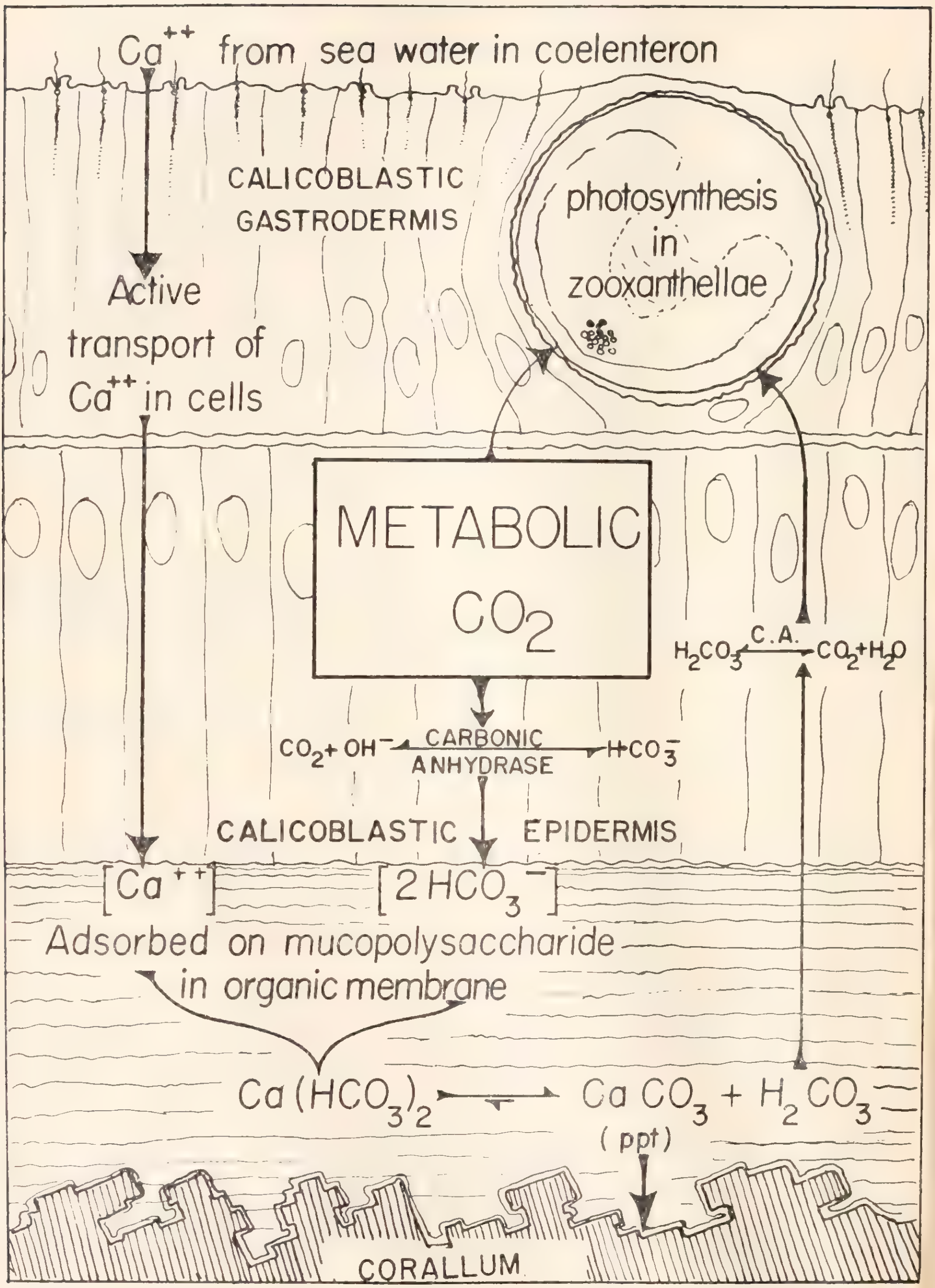
Ca++ from sea water in coelenteron
CALICOBLASTIC GASTRODERMIS
photosynthesis in zooxanthellae
Active transport of Ca++ in cells
METABOLIC CO2
H2CO3 C.A. CO2+H2O
CO2+ OH− CARBONIC ANHYDRASE H+CO3−
CALICOBLASTIC EPIDERMIS
[Ca++] [2 HCO3−]
Adsorbed on mucopolysaccharide in organic membrane
Ca(HCO3)2 ⇌ Ca CO3 + H2 CO3
(ppt)
CORALLUM

FIGURE 5. Diagram to show possible pathways of calcium and carbonate during calcificatio in a reef-building coral. A diagrammatic cross-section of the calicoblastic body wall at th base of the polyp is shown but the parts are not drawn to scale. The coelenteron and th

was sufficient to cause complete inhibition of carbonic anhydrase activity in coral homogenates as measured by the method of Meldrum and Roughton (1933). It is obvious that neither zooxanthellae nor carbonic anhydrase in themselves are essential to the calcification process, since this still goes on in the absence of one or both, though at a greatly reduced rate.

Discussion

The experiments described in this paper show that calcium deposition by madreporarian corals and other calcareous reef-builders can be determined under a variety of controlled conditions. The methods used here constitute a first step in the development of an accurate procedure for the rapid measurement of calcification rates applicable to further experimental studies of the physiology of skeletogenesis in corals.

The question arises as to whether coral growth rates determined under laboratory conditions can be compared to those found on the open reef under natural conditions. Since the experiments described above were not designed to test this, we are now conducting field studies, using a modified technic which will be described in a subsequent paper. Preliminary results show that calcification rates of coral are somewhat higher on the open reef than reported here, and that our standard light source was too bright for optimal coral growth. We have evidence that this latter factor accounts for the small and sometimes insignificant dark-light growth differences observed in some coral species as shown in Table I; *i.e.,* high light intensities could partially inhibit coral growth. The quantitative relationship of light intensity and other factors with coral growth is now under investigation.

A working hypothesis has been developed to help to interpret some of our results and to delineate the role played by the zooxanthellae and carbonic anhydrase in skeletogenesis of the reef-building madreporarian corals. To be satisfactory, such a hypothesis must account for: 1) the species-specific morphology of the skeleton; 2) its formation external to the body proper; 3) its chemical composition which is over ninety-nine per cent $CaCO_3$ and less than one per cent $MgCO_3$ (Vinogradov, 1953); and 4) the crystalline nature of the mineral matter which is nearly pure aragonite, according to Meigen (1903) and Chave (1954).

The calcification process is considered as a reaction in which Ca^{++} and $CO_3^{=}$ are brought to the calcification centers by separate pathways. The weight of histological evidence now indicates that the mineralization process occurs outside the calicoblastic epidermis (Matthai, 1918; Hayashi, 1937; Goreau, 1956) which secretes an organic matrix that may act as a template on which the final stages of skeletogenesis take place. It is of interest that this organic matrix contains an acid mucopolysaccharide-like substance (Goreau, 1956). This gives rise to the possibility that Ca^{++}, taken up from sea water and transported across the body wall to the external surface of the calicoblast, is adsorbed by ion exchange on an acidic space lattice provided by the mucopolysaccharide in the organic matrix. Here

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flagellated gastrodermis containing a zooxanthella are shown at the top of the figure, the calicoblastic epidermis is in the middle and the organic membrane with crystals of calcareous matter are at the bottom. The boring algae, the effects of which are problematical, have been omitted for simplicity. The direction of growth is upward, *i.e.,* calcium deposition is in a downward direction.

the Ca^{++} combines with HCO_3^- by the following reaction:

$$(1)\ Ca^{++} + 2HCO_3^- \rightleftharpoons Ca(HCO_3)_2.$$

The unstable product of this reaction then breaks down:

$$(2)\ Ca(HCO_3)_2 \rightleftharpoons CaCO_3(ppt) + H_2CO_3,$$

with the formation of calcium carbonate and carbonic acid. As long as calcium is not a limiting factor, the rate of formation of calcium carbonate will depend on the rate with which the carbonic acid is removed from the site of calcification. This can be accomplished through the fixation of CO_2 by photosynthesizing zooxanthellae and/or the action of carbonic anhydrase. The proposed scheme is summarized in Figure 5. It is expected, therefore, that if the zooxanthellae are prevented from photosynthesizing by keeping the coral in darkness, or if the algae are completely removed, the velocity of calcification will decrease, due to slowing down of reaction (*2*). Since carbonic anhydrase has an action which is, in this respect, physiologically equivalent to that of the zooxanthellae, the inhibition of the enzyme will also result in a slowing down of the calcification rate. The greatest decrease occurs when the corals are kept in darkness in the presence of a carbonic anhydrase inhibitor. The fact that calcification still goes on under these conditions simply shows that neither the enzyme nor the algae determine the basic calcification reaction, but that they can exert a strong influence on its over-all rate. This is in agreement with the work of Wilbur and Jodrey (1955) who showed that carbonic anhydrase does not affect shell calcification in the oyster unless the rate is limited by one of the following reactions:

$$(3)\ CO_2 + H_2O \rightleftharpoons H_2CO_3:$$

or

$$(4)\ CO_2 + OH^- \rightleftharpoons HCO_3^-,$$

hence the enzyme cannot be a primary factor in calcification as was previously assumed by Stolkowsky (1950) for mollusk shells.

An interesting problem arises from our data on calcification rates of reef corals from which the zooxanthellae had been removed. The second part of Table II shows that in darkness normal corals calcify from two to three times faster than corals which have lost their zooxanthellae. This suggests that the presence of these algal symbionts, even when not photosynthesizing, may have a potentiating effect on the calcification rate of the coral host. It is thus considered possible that the zooxanthellae can exert a general stimulant effect on the host's metabolism, mediated through a vitamin or hormone-like factor. This function of the zooxanthellae would to some extent be independent of the photosynthetically controlled "janitorial" activities of these algae which result in the assimilation of the animal host's metabolic waste products. It is hoped that work now in progress will provide more evidence for this interesting possibility.

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SUMMARY

1. A method is described for the accurate measurement of calcification rates in reef-building corals under various controlled conditions, using calcium-45 as tracer.

2. At the temperatures of the experiments, there was a slow but appreciable isotopic exchange between the coral skeleton and sea water. There are indications that this is considerably less in living coral where the tissue forms a barrier against such exchange.

3. In many of the reef-building corals tested so far, the calcification rate was significantly lowered by the exclusion of light.

4. The calcification rate of reef corals grown in darkness for prolonged periods of time to remove the zooxanthellae is considerably reduced and seems independent of the light intensity.

5. Variations in the growth rates of different parts of coral colonies were measured. The existence of growth gradients was demonstrated in a number of species.

6. Calcium uptake was greatly reduced on the addition of Diamox, a specific carbonic anhydrase inhibitor. In those species tested, the effect of carbonic anhydrase inhibition and exclusion of light was in the same direction. In the presence of complete inhibition of carbonic anhydrase there was still an uptake, even in darkness.

7. It was concluded that the effect of light on reef coral growth is in part mediated through the zooxanthellae. The decreased calcification rates of reef corals in darkness, in the absence of zooxanthellae or in the presence of a carbonic anhydrase inhibitor suggest that the rapid calcification of these corals may be dependent on efficient removal of H_2CO_3.

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THE REGULATION OF WATER AND SALT BY THE FIDDLER CRABS, UCA PUGNAX AND UCA PUGILATOR

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Ionic and osmotic regulation in decapod Crustacea are the result of selective ionic absorption and excretion through several routes (Prosser *et al.*, 1950; Robertson, 1953). The gills have been implicated as the primary site of absorption (Huf, 1936; Krogh, 1938; Webb, 1940; Koch, 1954; Gross, 1957) but the alimentary tract may also be important (Burger, 1957). The antennary glands are considered the chief organs of selective excretion (Nagel, 1934; Webb, 1940; Robertson, 1949; Prosser *et al.*, 1955; Burger, 1957). The cellular mechanisms of ionic absorption and excretion in crustaceans are poorly understood and hypo-osmotic regulation has been less extensively studied than hyper-osmotic regulation.

Recently (Prosser *et al.*, 1955) it has been shown that *Pachygrapsus crassipes*, when maintained in 170% sea water (S.W.) excretes a urine higher in Mg but significantly lower in Na than animals in normal sea water. The Na which is not excreted in the urine may be stored in tissues for short periods (Gross, 1958) or may be excreted by extra-antennary gland routes, as suggested by the finding (Gross, 1957) that salt exchanges, as measured by electroconductivity methods, occur in the gill chamber of *Pachygrapsus*.

Since Jones (1941) had shown that *Uca crenulata* is a stronger hypo-osmotic regulator than *P. crassipes*, studies were undertaken on several species of Atlantic coast *Uca* to determine their ability to excrete Na by extra-antennary gland routes. In a preliminary survey it was found that *Uca minax*, *U. pugilator* and U. *pugnax* all show hypo-osmotic regulation and reduced urine Na in concentrated sea water. These properties were not found in *Callinectes* and *Carcinus*. The object of the present paper is to report a detailed study of the response of the body fluids and tissues of *Uca pugnax* and *U. pugilator* to prolonged exposure to concentrated sea water.

MATERIALS AND METHODS

Uca pugnax and *U. pugilator* were acclimated to 175% sea water during three-day periods by increasing the concentration of the sea water 25% per day. The crabs were held at 175% sea water in large finger bowls containing a small amount of the bathing medium for 2 to 4 days after reaching this concentration. They were not fed in the laboratory but the sea water in the bowls was changed daily. Usually crabs were used within 7 to 14 days after collection. Some of the variability in the experiments may be attributed to the starvation of the animals and their varied nutritional states upon collection. Greater experimental variability is found between different batches of crabs than between the sexes of the two species.

Urine was collected from single animals by mounting the crab, caudal end down, on a microscope stage, attaching one wire from a Harvard inductorium to the mouth and stimulating the opercular region at the base of the antenna with the other. A small capillary drawn out at the end was simultaneously placed near the opercular covering. Usually moderate shocks resulted in the expulsion of urine, as much as 10–20 microliters from a single crab. Urine from three crabs was generally pooled on a piece of Parafilm which was kept in a high humidity chamber.

Blood was collected in the manner described for *Pachygrapsus* (Prosser *et al.*, 1955).

Gill fluid was collected through small openings made in the gill plate prior to the experiment. Care was taken to prevent bleeding at the time the openings were made. After exposing crabs to isotopic solutions for a 12-hour period, the animals were exposed to non-isotopic sea water for 15 minutes and transferred to dry finger bowls for 30 minutes before removing gill fluid by fine capillaries through the gill plate openings.

Stomach fluid was collected in capillaries from excised stomachs.

Some studies were performed with isotopic Na^{24}. This ion was obtained with Na_2CO_3 as the carrier and was initially made up in a small amount of distilled water. Ten-ml. aliquots of this highly active sample, containing 0.1–0.2 mc./mg., were placed in finger bowls containing 490 ml. of the appropriate sea water. Crabs were exposed to these isotope solutions for 12–18 hours before sampling. Exploratory experiments had shown that the relative specific activity of the serum of crabs remained nearly constant after 12 hours during the period of sampling.

Routinely, samples of blood, urine, gill fluid and stomach fluid were pooled from three animals for analysis. Twenty-five microliters of such a sample were added to 10 ml. of glass-distilled water in small Pyrex tubes. From this solution Na^{24} counts were made with a well counter and scintillation tube. Sodium, K, Ca and Mg were analyzed by flame photometry in a conventional manner using the Beckman flame attachment with a photomultiplier tube. Chloride was analyzed by the method of Schales and Schales (1941), SO_4 by the method of Nalefski and Takano (1950) and NH_4 by the method of Russell (1944).

Osmotic determinations were made on all fluid samples, using the Jones method (1941) as modified by Gross (1954).

In those studies where Na^{24} counts of tissue were made, tissues were removed to Parafilm, weighed, placed in test tubes with the Parafilm and counted in the same manner as were the fluids. Counts were expressed per 25 mg. of wet tissue.

RESULTS

Several preliminary experiments were performed to test our methods and to establish optimum levels for Na^{24} use. Table I presents the results from our two most extensive experiments for osmotic and ionic analyses of several fluids from crabs in 100% and 175% sea water. The measure of variability is the standard error. Significant differences between 100% and 175% groups were found for all components of serum except Mg, K, Ca and NH_4; for urine components except Ca, NH_4 and Cl; for gill fluid components except NH_4, and for components of stomach fluid except K and osmotic concentration.

A statistical evaluation of the difference between the analytical values (from

TABLE I

Osmotic and electrolyte concentrations in Uca expressed as mM/L

Fluid	No. crabs	Osmotic conc.*	Na	Mg	K	Ca	NH_4	Total mEq.$^+$	Cl	SO_4	Total mEq.$^-$
For Crabs in 100% S.W.											
Serum	28	.497	328	46	11	16	20	483	537	42	621
		±.012	±4.40	±2.55	±0.32	±1.35	±1.28		±7.75	±1.26	
Urine	23	.583	276	108	16	17	75	617	622	47	716
		±.014	±17.4	±11.2	±1.10	±0.89	±7.2		±25.8	±1.90	
Gill fluid	28	.506	314	64	10	12	18	494	569	36	641
		±.011	±9.73	±4.63	±0.50	±0.41	±2.04		±6.99	±1.96	
Stomach fluid	13	.758	335	101**	17	31	63	679	542	143	828
		±.036	±21.1	±21.2	±0.88	±3.19	±3.84		±17.7	±8.52	
For Crabs in 175% S.W.											
Serum	33	.587	375	55	15	14	21	549	574	49	672
		±.011	±9.1	±3.64	±0.48	±0.61	±1.88		±6.96	±1.11	
Urine	33	.683	218	255	20	20	116	904	704	120	944
		±.012	±18.18	±12.9	±0.71	±1.77	±7.7		±14.1	±4.89	
Gill fluid	33	.860	503	123	15	19	18	820	855	60	975
		±.023	±6.56	±6.69	±0.39	±0.31	±2.19		±9.40	±3.13	
Stomach fluid	18	.828	393	167	16	22	43	830	704	111	926
		±.015	±7.87	±12.6	±0.48	±0.65	±3.34		±37.0	±5.33	
Composition of S.W. used for experiments											
100% S.W.	—	.560	397	88	9	12	0	606	576	22	620
	—	.750	579	139	17	20	0	914	941	29	999

* Equivalent moles of NaCl; stomach average of 10 crabs.
** Average of 8 crabs.

Table I) of each of the fluids from crabs within the same medium is given in Table IV. The different fluids from animals within the same medium are as quantitatively distinct as are the same kinds of fluids from crabs in the two different media. For example, serum and urine from crabs in 100% sea water are as different as sera from

TABLE II

Analysis of Na^{24} counts in Uca tissues. Counts per 25 mg. wet weight

	100% S.W. Aver. cts.	175% S.W. Aver. cts.	% change 175%/100%	P values 100 vs. 175	P values 100 vs. sera	P values 175 vs. sera
Serum	3891.9	4365.1	112	<.01		
Muscle	1243.43	2035.06	166	<.005	<.005	<.005
Mid-gut gland	2020.95	1507.44	75	<.005	<.005	<.005
Stomach	2663.85	3508.3	132	<.005	>.050	<.005
Gill	5000.96	3576.9	72	<.005	<.01	<.005
Heart	1819.1	2122.36	117	>.100	<.005	<.005
Intestine	948.62	1132.15	119	>.050	<.005	<.005

crabs in normal and concentrated sea water. This finding emphasizes the existence of homeostatic mechanisms in this group of crabs. The ability of these crabs to maintain their sera hypo-osmotic to the medium in both normal and concentrated sea water as shown by the osmotic concentration, appears to be shared with other members of the grapsoid group (Robertson, 1953). More striking is the finding that crabs in both types of media produce a urine which is hypertonic to the serum. The data from Tables I and IV show that the crabs regulate all serum ions in concentrated sea water and all but Ca in normal sea water. With the exception of Na all other electrolytes occur in higher concentrations in urine than in serum. Since the degrees to which these ions are concentrated in the urine varies in crabs from the same medium and between the two media and also varies for the different ions, it is probable that their concentration is a result of secretion or selective ion reabsorption.

Table I indicates that the gill fluid from crabs in 175% sea water is hyper-osmotic to the medium, the serum and the urine. That this hypertonicity results from water and solute absorption as well as solute secretion will be apparent later. Directly related to the gill fluid hypertonicity is the urine Na concentration which is significantly lower (Table IV) in crabs in concentrated than in normal sea water. And while this result is not unexpected (Prosser *et al.*, 1955) it indicates the extra-antennary gland excretion of this ion, possibly through the gills, and hence its association with gill fluid hypertonicity.

TABLE III

Relative specific activities for crabs in normal and hypertonic sea water

Fluid	100% S.W.				175% S.W.				P Values
	No. crabs	Na mEq./L.	Counts per min.	RSA CPM/ Na^{23}	No. crabs	Na mEq./L.	Counts per min.	RSA CPM/ Na^{23}	RSA_{100} vs. RSA_{175}
Serum	13	349	2335	6.7	18	403	2955	7.8	0.01
Urine	13	258	2144	6.6	18	210	1599	7.6	0.05
Gill fluid	13	320	1099	3.7	18	486	2487	4.8	0.01
Stomach fluid	13	346	1816	5.8	18	393	2111	5.3	0.10

The stomach fluid of crabs in 100% sea water (Table I) is marked by its significant hypertonicity to serum, urine and gill fluid. Its ion content is different from serum except for Na and Cl; from urine except for Na, Mg and K and from gill fluid except for Na, Mg and Cl. The stomach fluid from crabs in 175% sea water is hypertonic to serum and urine but not to gill fluid. Its ion content is greater than that of serum for all ions except Na and K; stomach fluid is more concentrated than urine except for Ca, Cl and SO_4 and more concentrated than gill fluid except for K. Both water and solute absorption probably occur from the stomach and the distribution of electrolytes in the stomach fluid makes some secretion into the gut probable.

Fluid/serum ratios have been summarized in Figure 1 for osmotic concentration and the electrolyte values. The extent to which the urine/serum ratio (U/S) departs from unity has been used as a measure of antennary gland regulation (Prosser

TABLE IV

Probability values of analyses

Fluid	Osmotic conc.	Na	Mg	K	Ca	NH_4	Cl	SO_4
	A. Comparison of fluids of crabs in 100% and 175% S.W.							
Serum	<.01	<.01	=.05	>.10	>.10	>.50	<.01	<.01
Urine	<.01	<.01	<.01	<.01	>.05	>.10	>.50	<.01
Gill fluid	<.01	<.01	<.01	<.01	<.01	>.50	<.01	<.01
Stomach fluid	>.10	<.02	<.02	>.10	<.01	<.01	<.01	<.01
100% S.W.	B. Comparison of fluids from crabs in the same medium							
Serum vs. urine	<.01	<.01	<.01	<.01	>.50	<.01	<.01	<.02
Serum vs. gill fluid	>.50	>.10	<.01	>.10	<.01	>.10	<.01	>.02
Serum vs. stomach fluid	<.01	>.50	<.02	<.01	<.01	<.01	>.50	<.01
Urine vs. gill fluid	<.01	>.05	<.01	<.01	<.01	<.02	>.05	<.01
Stomach fluid vs. urine	<.01	=.05	>.50	>.10	<.01	>.10	<.02	<.01
Gill fluid vs. stomach fluid	<.01	>.10	>.05	<.01	<.01	<.01	>.10	<.01
175% S.W.								
Serum vs. urine	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01
Serum vs. gill fluid	<.01	<.01	<.01	>.10	<.01	>.10	<.01	<.01
Serum vs. stomach fluid	<.01	>.10	<.01	>.02	<.01	<.01	<.01	<.01
Urine vs. gill fluid	<.01	<.01	<.01	<.01	>.10	<.01	<.01	<.01
Stomach fluid vs. urine	<.01	<.01	<.01	<.01	>.50	<.01	>.50	>.10
Gill fluid vs. stomach fluid	>.10	<.01	<.01	>.10	<.01	<.01	<.01	<.01
100% S.W.	C. *Comparisons of ratios of fluids from crabs in the same medium							
U/S vs. one	<.01	<.02	<.01	<.01	>.10	<.01	=.01	<.01
SW/S vs. GF/S	<.01	<.01	<.01	>.02	>.50	<.01	>.10	<.01
SW/S vs. SF/S	<.02	<.01	>.10	<.01	<.01	<.01	>.10	<.01
175% S.W.								
U/S vs. one	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01
SW/S vs. GF/S	<.01	<.01	>.10	<.02	>.50	<.01	>.5	<.01
SW/S vs. SF/S	<.01	<.01	>.05	=.01	>.05	<.01	>.1	<.01

* See Figure 1 for meaning of ratios.

et al., 1955). When the U/S ratios for crabs in 100% sea water are compared with those from 175% sea water only the ratios for osmotic concentration, K and Cl are found to be alike; the 175% sea water U/S ratio for Na is lower and all others higher than the corresponding 100% sea water values. The considerable regulation exhibited by the antennary glands of these crabs in normal sea water (Tables I and IV) is increased under the stress of concentrated sea water, particularly for Mg, NH_4 and SO_4.

Because both gills and stomach have a direct contact with the external medium and appear to be the most likely sites of exchange of water and salts with the medium, it is reasoned that the extent to which the gill fluid/serum (GF/S) and the stomach fluid/serum (SF/S) ratios deviate from the sea water/serum ratio should provide a measure of the absorptive and secretory capacities of gill and stomach tissues. These ratios are presented in Figure 1, and the statistical significances of a variety of internal comparisons (for example, GF/S with SF/S ratios from crabs in 100% sea water) are given in Table IV.

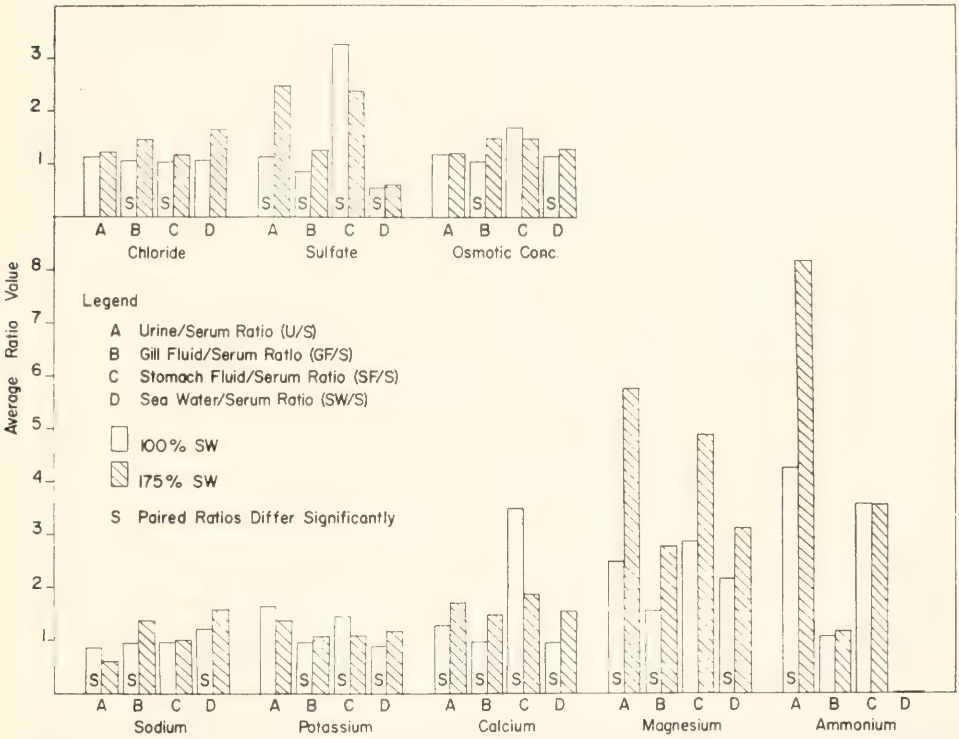

FIGURE 1. Ratios of the osmotic and electrolyte concentrations of fluids from crabs in 100 and 175% sea water. Statistical significance was attributed to P values of 0.02 or less.

In a few experiments crabs were exposed to sea water containing Na^{24}. The same quantity of the isotope was added to equal volumes of 100% and 175% sea water. Since the Na^{23} concentration of the 175% sea water was greater than that of the 100% sea water, a factor was used to correct the counts obtained from fluids and tissues of crabs in the concentrated sea water to make them comparable to those from normal sea water. This correction factor was obtained by dividing the Na^{24}/Na^{23} ratio in 100% sea water by the Na^{24}/Na^{23} ratio in 175% sea water. Multiplying the counts from the fluids and tissues from crabs in 175% sea water by this factor gave the corrected counts. Approximate isotopic equilibrium was attained in blood and urine in both groups of crabs after 12 hours. Isotopic analyses of tissues were made in a number of experiments and the assumption was made that these too had attained isotopic equilibrium. The results of the tissue studies are summarized in Table II. The high Na^{24} count in the gills from crabs in 100% sea water implies that these are the primary means of Na entrance or else that Na cannot be excreted as rapidly as it enters and dams up in this tissue. Since Na^{24} counts increased more in muscle (67%) and stomach tissues (32%) relative to the increase in sera in going from 100% sea water to 175% sea water, these tissues may serve for Na storage during the stress of high serum Na.

Only gill and mid-gut gland tissues from crabs in 175% sea water had lower Na^{24} counts than their 100% sea water counterparts; serum, muscle and stomach all had higher counts while heart and intestine were not significantly different. With the exception of the gill tissue from the crabs in 100% sea water the tissues of both groups had lower counts than their sera, which is in part an indication that the Na^{24} is restricted primarily to the extracellular space. The fact that in tissues from the 175% sea water animals, the counts from mid-gut gland and gills were proportionately lower while those of muscle and stomach were proportionately higher with respect to their sera than the same tissues from the 100% sea water crabs, indicates a differential tissue response to the Na stress.

A study of the rate at which Na^{24} can penetrate these crabs in 100% and 175% sea water has shown (Green and Harsch, 1958) that the isotope enters the crabs in the concentrated sea water more readily. This finding, coupled with the low Na^{24} counts in gills and mid-gut gland shown in Table II, affords evidence that these tissues are concerned with the excretion of Na under the conditions of these experiments.

When a comparison is made of the relative specific activities (RSA) (counts per minute/meq of Na^{23}/L) for serum, urine, gill fluid and stomach fluid for the two groups of crabs, as summarized in Table III, only serum and gill fluid values are found to be significantly different. The high RSA value of serum for the crabs in 175% sea water indicates a greater exchange rate of Na^{24} for Na^{23} as compared with normal sea water. The higher RSA value in the gill fluid is interpreted to mean that the crabs in hypertonic sea water excrete more Na by the gills than do crabs in normal sea water.

Discussion

The osmotic concentration data of Table I indicate that in both normal and concentrated sea water *Uca* is a hypo-osmotic regulator; sera of 100% sea water crabs were 12% lower in osmotic concentration and of 175% sea water crabs 22% lower than their respective media. Hypo-osmotic regulation occurs in crabs which spend much time out of water (Jones, 1941) and in shrimps and prawns (Parry, 1954). Ionic regulation in *Uca* is quantitatively different in the two media. In 100% sea water the serum concentrations as per cent of medium concentrations are: Na, 83; Mg, 52; K, 122; Ca, 133; Cl, 93; SO_4, 191; while in 175% sea water they are: Na, 65; Mg, 40; K, 88; Ca, 70; Cl, 61; SO_4, 169. In the concentrated sea water each ion is proportionately less concentrated in serum relative to the medium than in normal sea water; however, the extent to which the ions are regulated, as measured by the per cent increase in serum concentrations in 175% sea water relative to the sera concentrations in 100% sea water, is, in order of decreasing order of regulation: Cl, 7; Na, 14; Ca, 14; SO_4, 17; Mg, 20; K, 36. *Uca* differs from

Pachygrapsus crassipes (Prosser *et al.,* 1955) under similar conditions, especially in the greater ability of the fiddler crab to regulate Na. The osmotic concentration of the 100% sea water crabs approximates that of *Pachygrapsus marmoratus* as measured by Robertson (1953), as do the K, Ca, and SO_4 values relative to Cl, while Mg values in *Uca* are relatively higher.

A cation deficit of 12% exists in the serum of crabs in 100% sea water; a deficit of 10% in crabs in 175% sea water. The urine cation deficit is smaller in both groups. The serum deficits are attributed to organic cations. The lower cation deficit in urine than in serum is associated with the higher urine concentration of ammonia; however, if the NH_4 excreted by the antennary glands is subtracted from the total cation deficit, the cation deficit is still smaller than that in serum.

Tentative conclusions can be drawn concerning the formation of urine, gill fluid and stomach fluid in *Uca.* The urine in both normal and concentrated sea water has a higher osmotic concentration and a higher total electrolyte concentration than serum. This ability to produce a blood hyper-osmotic urine is one means of keeping the blood hypo-osmotic to the medium. *Pachygrapsus crassipes* failed to show a hyper-osmotic urine (Prosser *et al.*, 1955). *Uca* appears to spend more time out of water than *Pachygrapsus* and may be a better hypo-osmotic regulator, partly because of its ability to produce hyper-osmotic urine.

TABLE V

pH of Uca urine

Treatment of crabs	Crabs in 100% S.W.	Crabs in 175% S.W.
Equilibrated to medium for 3 days	6.92±.15*	7.16±.10
Equilibrated to medium for 4 weeks	6.38±.11	6.42±.11

* Standard error. The pH was measured with the Beckman micro-glass electrode. Urine of crabs equilibrated at the same time was not significantly different. Differences in the urine pH of crabs in the same media for different lengths of time were real.

It was not feasible to obtain true urine volumes, and excretion of solutes which are unlikely to be transported actively was not studied. The urine/serum ratios (U/S) differ for different ions and are maximal for NH_4 (4 and 8 in 100% and 175% sea water). If NH_4 were excreted by simple filtration, marked reabsorption of all other ions would be required to give such high NH_4 values; hence it is probable that NH_4 is either secreted or its excretion is accelerated by acidification of the urine. This latter alternative appears unlikely from the pH data presented in Table V. The U/S ratio is next highest for Mg, increases proportionately in the concentrated sea water. The high U/S ratios for Mg and SO_4 in (175% sea water) could result from marked reabsorption of water and other ions (except NH_4); they could indicate secretion of Mg and, at least in 175% sea water, also of SO_4.

The treatment of Na by the antennary glands is unique. Its U/S ratio is less than one in normal sea water and is decreased in 175% sea water. This reduction in urine Na was found in *Pachygrapsus* (Prosser *et al.*, 1955) and has been seen in another semi-terrestrial genus, *Ocypode* (Gifford, unpublished data). Reduced urine Na in concentrated sea water is not necessary for hypo-osmotic regulation, however, since it does not occur in hypo-osmotic shrimps and prawns (Parry, 1954). The decreased urine Na in 175% sea water could result from reduced secretion or increased reabsorption. In *Pachygrapsus* urine Na was not reduced in 175% sea water which lacked Mg (Prosser *et al.*, 1955); injection of extra Mg into land crabs in 100% sea water reduces urine Na (Gifford, unpublished data). In *Uca* in 175% sea water Mg excretion increases more than Na excretion decreases when both are compared with responses in 100% sea water. It seems probable from these observations that Mg secretion interferes in some way with Na secretion. Filtration and reabsorption of Na might serve a useful function in causing water absorption. However, one would expect such Na reabsorption to be associated with some Cl or SO_4 absorption; this does not appear to be the case. If the Na were reabsorbed by exchange with Mg one would expect two Na ions to be exchanged for one of Mg; the finding for 175% sea water was 1.3 ions of Mg for each ion of Na. Hence on a quantitative basis it is difficult to attribute an increased Na reabsorption to an increased Mg excretion. On an energetic basis Na reabsorption seems improbable. If the crab needs to remove Na in 175% sea water and is able to filter it in the kidney, why would this Na be reabsorbed against a Na gradient, using energy for this purpose, only to be secreted at another site using energy a second time? It is possible that Na is exchanged for NH_4 or for hydrogen ions, in which case its absorption would serve a useful function. By exclusion, active secretion of Na is indicated, a process which is reduced under a high Mg load.

The U/S ratios for K, Cl and Ca are slightly above one and increase slightly in 175% sea water. Since the ionic gradients of these elements are from urine to plasma (presumably because of water reabsorption) the differences among them could result from differences in back permeability among these ions. Rather than postulate secretion of all ions, it seems more reasonable that there is filtration coupled with reabsorption of Na and water and some secretion of NH_4 and Mg (possibly also SO_4).

The composition of fluid from the gill chamber indicates a combination of diffusion and secretion and a mixing with sea water. The osmotic concentration is intermediate between serum and 100% sea water; it may be as high or higher than 175% sea water. This could mean outward secretion of some ions or absorption of water. Ammonia in gill fluid is intermediate between serum and the medium, hence NH_4 appears to be lost from the gills only by diffusion.

Sodium concentration in gill fluid is similar to Na in serum in 100% sea water, but is much higher in 175% sea water. In both concentrations it is lower than in the medium. The gill fluid specific activity is significantly higher in 175% than 100% sea water while the gill tissue has significantly fewer Na^{24} counts in 175% than 100% sea water. It is concluded that active secretion of Na occurs in the gills, at least in 175% sea water. The diffusion gradient for SO_4 is outward in 100% sea water but in 175% sea water the SO_4 in gill fluid is higher than in either serum or medium; hence there might be some SO_4 secretion along with Na.

Magnesium and Cl in gill fluid resemble Na in being close to serum levels in 100% sea water and higher than serum but lower than 175% sea water. These gradients could result from diffusion, secretion (in 175% sea water) or from water absorption. Potassium is similar in serum, gill fluid and medium; Ca in gill fluid is similar to both media, lower than serum in 100% and slightly higher in 175%

sea water. It is difficult to see how these concentrations could be so similar if there were much absorption of water. The relative importance of differences in permeability, in secretion and of water uptake by the gills cannot be evaluated from the present data. However it appears that the gills are important in ionic regulation in *Uca* and that the univalent ions do not separate from the divalent ions in route of excretion as they do in marine teleosts.

It is probable that, like the lobster (Burger, 1957), *Uca* swallows some sea water, hence stomach fluid is a modification of sea water. Since NH_4 is absent from sea water and is higher in stomach fluid than in serum, gastric secretion of NH_4 is probable; however the concentration of NH_4 in stomach fluid is less than in urine, where more secretion is indicated, and greater than in gill fluid where NH_4 may be lost only by diffusion.

Sodium in stomach fluid is similar in concentration to Na in serum in both media but lower than sea water, especially 175% sea water, hence absorption of Na in the stomach is indicated. With the Na, water is also probably absorbed, as indicated by the higher osmotic concentration in stomach fluid than in sea water. Concentration of the other ions might then be established by different inward permeabilities. A less likely alternative would be absorption of sea water and then active secretion of the different ions. Sulfate in stomach fluid is so high that it may well be secreted. In any case there must be some absorption of ions other than Na along with water; presumably this is the source of the Mg which is excreted in such large amounts by the antennary glands (kidneys).

The significantly higher Na^{24} levels found in stomach and muscle tissues of crabs in concentrated sea water indicate that during Na stress these tissues may serve as repositories for Na, as indicated by Gross (1958). It is unlikely that this storage mechanism is confined to a single kind of ion or that it can account for ionic regulation in a concentrated medium for long periods of time.

In the absence of data on fluid volumes and kidney clearances, a tentative qualitative summary is as follows: Ammonia diffuses from the gills, is actively excreted in the stomach and very much concentrated in the urine. Sea water is swallowed, especially in 175% sea water, Na and water are absorbed, other ions to a less extent. Filtration occurs in the kidney although Mg and Na may be actively excreted; Na and water may be reabsorbed. In 175% sea water the heavy load of Mg excretion is coupled with decreased secretion or increased reabsorption of Na. Sodium (also probably SO_4) appears to be actively secreted by the gills, more in concentrated than in normal sea water.

The various fluids which have been measured represent steady-state concentrations resulting from diffusion and selective permeabilities combined with active transport, and fluxes can only be inferred.

SUMMARY

1. Analyses were made of the serum, urine, gill and stomach fluids for total osmotic concentration and the electrolytes Na, Mg, K, Ca, NH_4, Cl and SO_4 in *Uca pugnax* and *U. pugilator* when these two species were kept in 100% and 175% sea water.

2. For crabs in 100% sea water the serum electrolyte values for Na, Mg and Cl are lower and those for K, Ca, NH_4 and SO_4 higher than in the medium; for crabs

in 175% sea water the serum electrolyte values of Na, Mg, K, Ca, NH_4 and Cl are lower and only SO_4 higher than the values in the medium. The sera of crabs from both media are hypotonic to their saline environment.

3. The electrolyte values of sera from crabs in normal sea water differ significantly from the gill fluid electrolytes for Mg, Ca and Cl only; while similar sera values from crabs in concentrated sea water differ significantly for Na, Mg, Ca, Cl and SO_4. In all cases except for Ca from crabs in normal sea water the significant gill fluid electrolyte concentrations are greater than the corresponding sera values.

4. Crabs in normal and concentrated sea water maintain their stomach fluids more concentrated than the external medium. Sera electrolyte concentrations from crabs in 100% sea water are significantly lower than stomach fluid concentration for Mg, K, Ca, NH_4 and SO_4. In crabs from 175% sea water corresponding serum electrolyte significance is found for Mg, Ca, NH_4, Cl and SO_4.

5. All electrolytes are regulated by the antennary gland by crabs in the high salinity medium and all except Ca in the normal sea water; Mg and NH_4 are especially controlled by the antennary gland. In concentrated media the antennary gland excretion of Na is significantly lower than in normal sea water while the Mg excretion is markedly elevated.

6. Ammonia appears to be secreted by both the antennary gland and the stomach but its appearance in the gill fluid is attributed to diffusion.

7. Urine osmotic and electrolyte concentrations are significantly higher than the corresponding serum concentrations for animals in both media.

8. For crabs in 100% sea water the average fluid osmotic concentrations are equivalent to the following moles of NaCl: serum, 0.497; urine, 0.583; gill fluid, 0.506 and stomach fluid, 0.758; for crabs in 175% sea water the corresponding values are: serum, 0.587; urine, 0.683; gill fluid, 0.860 and for stomach fluid, 0.828.

9. By the use of Na^{24}, the relative specific activities of serum and gill fluid from crabs in 175% sea water are shown to be significantly higher than the corresponding serum and gill fluid values from crabs in 100% sea water while the RSA values of the urines are not significantly different. Na^{24} counts in gill tissue from the 175% sea water crabs are significantly lower than in the 100% sea water crabs. Active excretion of Na by the gills is indicated.

10. The low isotopic concentration of the mid-gut gland from crabs in concentrated sea water, comparable to that of gill tissue, suggests a Na secretory mechanism for this organ. The high isotopic Na concentrations found in muscle and stomach tissues of crabs in 175% sea water indicate that these tissues may be serving as storage depots during periods of serum Na stress.

11. The data show that the chief sites of entrance of water and electrolytes into these fiddler crabs are the stomach and the gills. They show that the chief sites of regulation are the antennary glands and the gills with some regulation by the stomach and possibly the mid-gut gland.

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STUDIES ON THE ROLE OF THE CORPUS ALLATUM IN THE ERI-SILKWORM, PHILOSAMIA CYNTHIA RICINI[1]

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The corpus allatum of insects has two known functions. In the developing insect, it furnishes a hormone which, in collaboration with the growth and differentiation hormone of the prothoracic glands (or their homologues), brings about larval molts. In the adult female, presumably the same corpus allatum hormone stimulates gonadal development, especially yolk deposition in the eggs. The latter effect has been demonstrated in a variety of species representing, among others, Orthoptera (Pfeiffer, 1939; Scharrer, 1946), Hemiptera (Wigglesworth, 1936), and Diptera (Thomsen, 1940, 1942; Vogt, 1941, 1943; Day, 1943). On the other hand, the adult ovaries of several representatives of Lepidoptera tested proved independent of the corpus allatum hormone (Bounhiol, 1942; Fukuda, 1944; Williams, 1946).

In another lepidopteran, the Eri-silkworm, *Philosamia cynthia ricini*, the corpus allatum of the newly emerged moth is 20 times larger than that of the last instar larva, an observation which suggests that this gland is functionally active in the adult of this species. In the course of experiments designed to demonstrate this physiological activity in adult *Philosamia*, a new role of the corpus allatum was discovered.

MATERIAL AND METHODS

Larvae of *Philosamia* were reared at around 25° C. Pupae from which the brain had been removed not later than 22 hours after pupation (artificially induced diapause) were used as test animals. Four to 6 corpora allata from donors of different stages were implanted into these diapausing pupae through a small hole in the dorsal integument of the second or third abdominal segment. The hole was then covered with a piece of integument and the wound was coated with melted paraffin. In some additional experiments, brains were implanted together with corpora allata; in others, corpora cardiaca were added, since they are known to store neurosecretory material originating in the brain. Following the implantation, the specimens were kept again at about 25° C. and were examined at appropriate intervals.

RESULTS

1. Implantation of corpora allata from adult donors

Implants of corpora allata from male or female donors whose adult age was 1–2 days, into diapausing pupae that had been deprived of their brains for two months,

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were effective in 9 out of 10 cases (Table I). Within 22–32 days after implantation the hosts underwent an additional pupal molt. These animals were unable to shed the old pupal cuticle by themselves, but molting fluid was present so abundantly that the old cuticle could be easily removed by forceps. The new pupal skin thus exposed was of normal color in the posterior half of the animal, but it appeared yellowish white in the anterior part. The imaginal discs of wings, antennae, and legs showed a very slight development toward the adult form while other organs displayed no sign of adult differentiation.

This result reveals two important effects of the corpus allatum of *Philosamia:* (1) the implants must have furnished juvenile hormone since the molt following their implantation was pupal rather than adult. This effect is in keeping with the known role of the corpora allata in a variety of insect species. (2) The implants, in addition to the juvenile hormone, must have furnished a principle which initiated molting in a diapausing host deprived of its brain. It was concluded that this molt-inducing hormone originated in the neurosecretory cells of the brain of the donor and was stored in its corpus allatum. An axonal transport of neurosecretory material produced in the insect protocerebrum has already been demonstrated in earlier investigations (Scharrer and Scharrer, 1944; Scharrer, 1952; M. Thomsen, 1954, and others). In many species, the neurosecretory material can be traced only as far as the corpora cardiaca which in these forms are considered as the main storage and release center of neurosecretory hormones. Therefore, corpus cardiacum implants and brain implants, either alone or in combination with corpus allatum implants, also were tested.

TABLE I

Implantation of endocrine organs isolated from adults

Endocrine organ	Number of implanted organs	Number of experimental specimens	Number of deaths or undeveloped cases	Number of adults	Number of second pupal instars
Corpus allatum	4–6	10	1	0	9 (90%)
Corpus cardiacum	4–6	10	0	1* (10%)	0
Brain-cardiaca-allata complex	3	15	3	0	12 (80%)

* The interval needed for its development was abnormally long.

When four to six corpora cardiaca were implanted into each of ten diapausing pupae, only one of the recipients emerged 47 days later, an interval much longer than that normally required for adult development. The other nine hosts remained unchanged. This result demonstrates at best only a minor role of the corpus cardiacum of *Philosamia* as a storage center for neurosecretory material.

Each of 15 diapausing pupae (417 days after their brain was extirpated) received three complexes of brain-corpora cardiaca-allata plus subesophageal ganglion. Three animals died. Twelve of the hosts pupated again within three weeks after implantation; none proceeded to become an adult moth. These results do not differ from those after the implantation of corpora allata alone.

2. *Implantation of corpora allata from pupal donors*

The pupae which furnished the corpora allata in this series had passed from 11 to 13 days in the pupal state. Again each of the diapausing hosts received six corpora allata. Seventeen out of 22 pupae thus operated upon differentiated quite normally into moths within 25 days after the implantation (Table II). The remaining five hosts remained pupae or died before showing any positive result. It is of interest that none in this group underwent a second pupal molt. Thus the result differs from that of the previous experiment in which adult corpus allatum implants had been used. One must conclude that pupal corpora allata contain only the hormone which stimulates the prothoracic glands, but are devoid of appreciable amounts of juvenile hormone.

The addition of pupal brains and corpora cardiaca to corpus allatum implants did not alter the outcome of the results. Twenty-one out of 22 diapausing animals receiving these grafts emerged after about 25 days; the remaining one died. Thus, none of these animals underwent an additional pupal molt.

TABLE II

Implantation of corpora allata isolated from pupae and larvae

Endocrine organ	Number of implanted organs	Number of experimental specimens	Number of deaths or undeveloped cases	Number of adults	Number of second pupal instars
Pupal donors:					
Corpus allatum	6	22	5	17 (77.3%)	0
Brain-cardiaca-allata complex	3	22	1	21 (95.4%)	0
Brain	3	23	4	19 (82.6%)	0
Larval donors:					
Corpus allatum (5th instar)	6	24	0	5* (20.8%)	19 (79.2%)
Corpus allatum (4th instar)	6	27	6	0	21 (77.8%)

* One specimen required an abnormally long interval.

In another group of test animals each of which received three pupal brains, emergence occurred after the same period of time in 19 out of 23 specimens. These results show that (a) implants of either pupal brains or pupal corpora allata furnish the hormone necessary for the initiation of adult differentiation, and (b) pupal corpora allata do not contain appreciable amounts of juvenile hormone.

3. *Implantation of corpora allata from larval donors*

Among 27 test animals which received corpora allata removed from fourth instar caterpillars two days before the next molt, 21 underwent a second pupal molt within 11 to 14 days. None showed adult differentiation. The result was somewhat different when the donors were fifth instars which had just entered the spinning stage. In this group 19 out of 24 test animals had another pupal molt while four became adult moths after a normal, and one after a prolonged, interval of time. It seems that in the last mentioned five cases the corpora allata had already ceased to secrete juvenile hormone.

4. *Extirpation of corpora allata from pupae*

Since the preceding experiments had demonstrated the presence of juvenile hormone in the corpora allata not only of larval but also of adult *Philosamia,* the question arose which role is played by these glands in the imago. A possible control over gonadal activity was tested by removing the corpora allata from pupae not older than 40 hours which were then allowed to complete their adult development. Twelve allatectomized specimens did not differ essentially from 20 sham operated controls. In each group about the same number of eggs became mature (Table III). In other words, in *Philosamia* ovarian function seems to be independent of the corpora allata. Future tests with biochemical methods will be needed to show whether or not the corpora allata in this species have a metabolic function.

Table III

Comparison of egg development in allatectomized and control females

	Number of specimens examined	Average number of eggs		
		Mature	Immature	Total
Allatectomized	12	128	150	278
Control	20	154	131	285

Discussion

The present experiments have revealed that in *Philosamia* brainless pupae can be induced to molt by the implantation of corpora allata. Depending on the stage of the donor, the molt caused may or may not be coupled with adult differentiation. Larval and adult corpora allata furnish enough juvenile hormone to render the ensuing molt of the test animal a second pupal molt. By contrast, pupal corpora allata lack effective doses of juvenile hormone. The type of molt occurring is, however, of less interest than the fact that molts can be induced at all by corpus allatum implants in cases where they would otherwise not occur. While it has been known for some time that corpora allata from larval and adult donors can furnish juvenile hormone, the present study offers the first evidence that corpus allatum implants can induce molting. Theoretically, the molt-inducing hormone present in the corpus allatum implants used in our experiments either could have originated in the corpora allata themselves, or it could merely have been stored there. The first possibility seems less likely. The reasons for assuming the second mode of action are as follows. In *Philosamia* as well as other forms of insects, neurosecretory cells of the brain are known to furnish a hormone which stimulates the prothoracic glands into releasing a molt-promoting hormone. It is also known that this neurosecretory material is transported along axons and stored at some distance from the site of origin. In a variety of species the storage and release center is the corpus cardiacum. In some species, including *Philosamia cynthia*, neurosecretory material has been observed to enter also the corpus allatum. However, the possibility that this gland stores neurosecretory material in appreciable amounts has never been tested experimentally with positive results. So far, the presence of neurosecretory material within the corpus allatum tissue has been interpreted as a possible morphological indication for the existence of an allatotropic action on the part of neurosecretory cells (E. Thomsen, 1954). The present study neither contradicts nor supports this view. However, judging from the result with pupal donors of *Philosamia*, juvenile hormone can be absent in corpora allata in which brain hormone is known to be stored. Therefore, one would have to assume that corpus allatum cells do not necessarily respond under all circumstances to stimulation by an "allatotropic hormone." Furthermore this factor may or may not be identical with the molt-inducing hormone.

The present study offers evidence that implants of corpora allata in *Philosamia* furnish brainless pupae with a sufficient amount of neurosecretory material to induce them to molt. It does not prove that in the intact animal the corpus allatum tissue serves as the main storage and release center of a hormone produced by the brain. The possibility exists that neurosecretory material which reaches the organ via the nervi corporis allati accumulates within the corpus allatum in gradually increasing amounts without being given off into the circulation. This situation would perhaps be comparable to the accumulation of juvenile hormone in the abdomen of adult males of *Platysamia* (Williams, 1956). Further experiments will be needed to determine whether in species such as *Philosamia* with inconspicuous corpora cardiaca the corpora allata indeed take over the main storage and release function.

The experimental demonstration of the presence of molt-promoting hormone in the corpora allata of *Philosamia* is paralleled by morphological data showing the existence of a corresponding neurosecretory pathway. The presence of neurosecretory material in the nervi corporis allati has been observed in *Bombyx* (Bounhiol, Gabe and Arvy, 1953, 1954; Kobayashi, 1957) as well as *Philosamia* (unpublished observations of the authors).

Whatever the mechanism of release of neurosecretory hormones under normal physiological conditions, the fact remains that, with the exception of the pupal stage, the corpora allata of *Philosamia* contain two hormones controlling post-embryonic development, the "prothoracotropic hormone" of neurosecretory origin and the "juvenile hormone" produced by the corpus allatum cells themselves.

SUMMARY

1. Pupae of *Philosamia cynthia ricini* in which diapause had been artificially induced by the removal of the brain, served as test animals for the effects of corpus allatum implants. Four to six corpora allata from donors in different stages induced molting in hosts which otherwise would have remained pupae. It was concluded that in *Philosamia* the corpus allatum, in addition to producing juvenile hormone, contains an appreciable amount of molt-inducing hormone furnished by neurosecretory cells of the brain. The interpretation is supported by the existence, in *Philosamia* as well as other insect species, of a neurosecretory pathway which links

the secretory part of the brain with the corpora cardiaca-allata and which permits the storage of hormones produced in the brain at some distance from the cells of origin. While in most species studied so far the main storage center is the corpus cardiacum, this role may have been taken over by the corpus allatum in *Philosamia.*

2. As might be expected, the molt induced may or may not be coupled with adult differentiation depending on the stage of the donor. Implants of corpora allata from adult or fourth instar larval donors caused an additional pupal molt because, in addition to molt-inducing hormone, they also supplied juvenile hormone to the host. By contrast, implants from pupal donors contained no appreciable amount of juvenile hormone with the result that they brought about an imaginal molt. Some of the fifth instar implants had the same effect as those from pupae, while others acted like tissues from fourth instars. It seems that during the fifth larval stage the change from activity to temporary inactivity of the corpus allatum cells occurs gradually. Thus implants of larval and adult corpora allata furnish two hormones controlling post-embryonic development, while pupal corpora allata contain only one, namely, the neurosecretory material derived from the protocerebrum.

3. Even though the presence of corpus allatum hormone has been demonstrated in glands from adult donors in the present experiments, the role normally played by this hormone in the adult moth is still unknown. Extirpation of corpora allata from female pupae of *Philosamia* did not prevent egg maturation in the resulting moths.

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BETA-GLUCOSIDASE OF THE MIDGUT OF THE SILKWORM BOMBYX MORI

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Contrary to a wide distribution of β-glucosidase in plants, the occurrence of this enzyme in insects seems to be rare, since neither utilization of β-glucosides nor presence of the enzyme activity has been often recognized in insects. Until recently, the demonstration of this enzyme in insects has been discussed on the basis of the utilization of β-glucosides in growth experiments (see the review by Lipke and Fraenkel, 1956). Studies of this enzyme from the enzymic points of view, however, have been lately carried out with the wood louse *Porcellio* (Newcomer, 1952, 1956) and the cockroach *Periplaneta americana* (Newcomer, 1954). Applying a highly sensitive fluorimetric method for β-glucosidase assay, Robinson (1956) has demonstrated the occurrence of the enzyme in the locust *Locusta migratoria,* the mealworm *Tenebrio molitor,* the water-boatman *Notonecta,* the cockroach *Periplaneta americana,* and the black aphis *Aphis fabae.* The occurrence of this enzyme has also been reported for the bean weevil *Callosobruchus chinensis,* the bean blister beetle *Epicauta gorhami,* the silkworm *Bombyx mori,* and the wild silkworm *Dictyoploca japonica* (Koike, 1954), and for the mealworm *Tenebrio molitor* (Fraenkel, 1955).

A few years ago the present authors became aware of the fact that the midgut homogenate of the silkworm is able to hydrolyze salicin, but the digestive fluid gave scarcely the same reaction. Recently, this problem was re-investigated to obtain more detailed results. This report is mainly concerned with the occurrence of β-glucosidase in the silkworm midgut, its characterization, and partial purification. A comparison of the enzyme activity of the normal larvae was also made with amylase-free mutants, and with jaundice-diseased larvae.

MATERIALS AND METHODS

Practical methods of obtaining midgut homogenates have been previously reported (Ito, Horie and Ishikawa, in press; Ito and Horie, in press). Homogenates made in water were used directly in some experiments, but the acetone powder made with midgut homogenates was used for most enzyme preparations. Midgut homogenates made from middle fifth instar larvae were dehydrated by mixing with 7 volumes of chilled acetone and the precipitates were collected in a Büchner funnel under suction. The precipitates were subsequently re-suspended in chilled acetone, then separated from acetone with funnel as above. The precipitates were washed by running alcohol-ether mixture (1:1) and brought to dryness in a vacuum desiccator. The dried, pale-yellow cake was ground in a mortar and the acetone powder thus made was used for enzyme tests. The powder was kept *in vacuo* at 5° C. at least for 8 months without any loss of β-glucosidase activity. Preparing the enzyme solution, the powder was suspended in water, allowed to stand for two hours at 5° C., and the supernate, obtained after centrifugation at 10,000 × g for 10 minutes, was used for the experiments.

Digestive fluid was collected from middle fifth instar larvae by applying a weak electric shock to them. The fluid was either used for enzymic measurements directly after dialysis against water at 5° C. for 48 hours, or after conversion to an acetone powder.

Enzyme activity was assayed by measuring the amount of glucose liberated from β-glucoside in the reaction system. Salicin was used as a substrate in most experiments, and cellobiose or phenyl β-glucoside in some. Unless otherwise indicated, each reaction mixture contained 200 μM citrate buffer (pH 5.4), 48 μM salicin and 1.0 ml. acetone powder solution (total volume 4.0 ml.) and was incubated at 30° C. for two hours. The reaction was stopped at intervals by adding an aliquot to $Ba(OH)_2$ and $ZnSO_4$, or Na_2WO_4. When cellobiose was used as the substrate, the reaction was stopped by Na_2WO_4 and H_2SO_4, and bakers' yeast then applied to the supernate of the reaction mixture in order to remove fermentable sugar. Glucose was determined mainly by the method of Hagedorn and Jensen (1923) and sometimes by Somogyi's procedure (1952).

Nitrogen was determined by the micro-Kjeldahl method.

RESULTS

Optimal pH range

The supernate obtained from an acetone powder suspension was incubated with various buffers at different pH levels. As shown in Figure 1, almost no alteration of pH optimum was found with different buffers. Optimal pH ranges were 5.0–6.2 for citrate and phosphate, and 5.2–6.4 for acetate. These ranges are more extended than those reported for other insects (Newcomer, 1952, 1954, 1956; Robinson, 1956) and for plants (Veibel, 1950). The enzymic activity was relatively high at a high pH level such as 7.0 or even 8.0, which has not been reported so far for other species of insects. The measurement also showed that β-glucosidase activity in borate buffer was not reduced to zero at pH 9.4. It has been known that the pH optimum of this enzyme is dependent on the source of the enzyme and to a minor degree on the substrate and the buffer solution (Veibel, 1950).

Velocity of hydrolysis

The relationship between enzyme concentration and velocity of hydrolysis is shown in Figure 2, where the enzyme concentration was doubled, respectively, from curve 3 to curve 1 (1:2:4). It is apparent that the rate of glucose liberation is proportional to enzyme concentration. Figure 2 also shows that the reaction proceeded at a uniform rate when enzyme concentration was relatively low.

In Figure 3 the effect of the concentration of the substrate on the enzyme activity is shown. The curves were plotted according to the procedure of Lineweaver and Burk (1934), *i.e.*, the inverse of the activity against the inverse of salicin concentration. The Km value (the Michaelis constant) is 0.013 M, which is in accord with the value reported for salicin (Veibel and Lillelund, 1938).

Inhibition by high temperature

The effect of high temperature on midgut β-glucosidase is shown in Table I. The supernate obtained from acetone powder suspension was treated at 40 to 70° C.

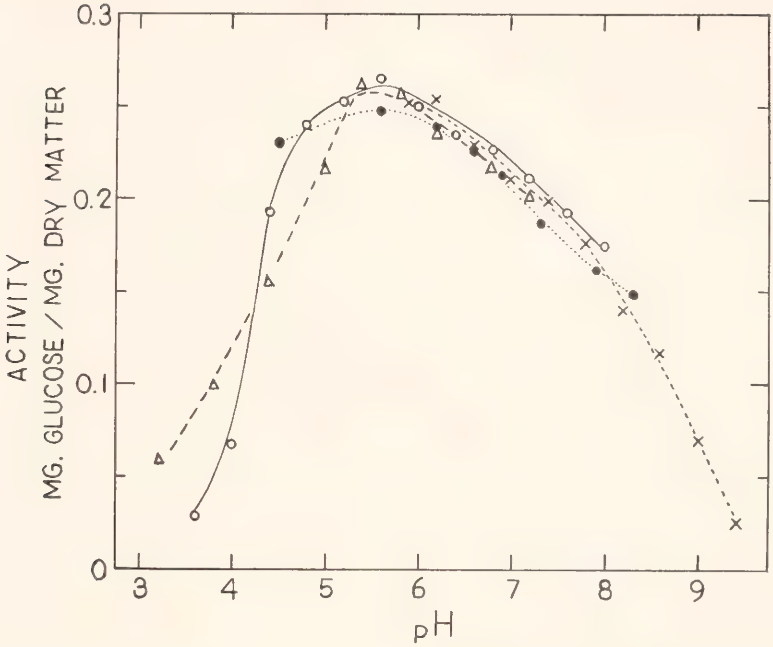
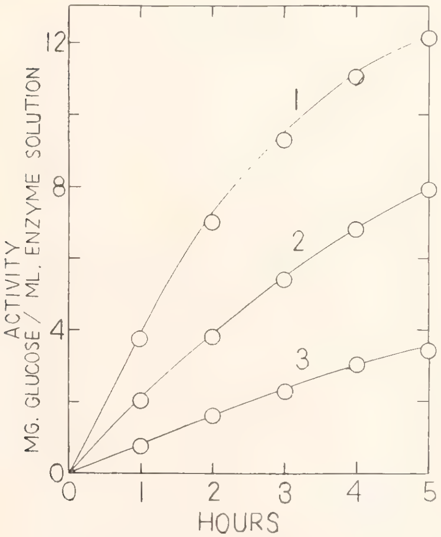


FIGURE 1. Relationship between pH and β-glucosidase activity. Phosphate buffer: ●..........●. Borate buffer: ×--------×. Citrate buffer: ○———○. Acetate buffer: △— — — —△.



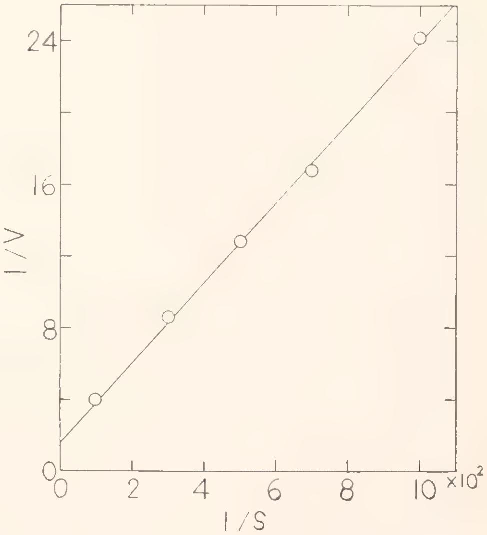


FIGURE 2 (left). β-Glucosidase activity as a function of time for different enzyme concentrations.

FIGURE 3 (right). Relationship between β-glucosidase activity and salicin concentration. Borate buffer (pH 6.0). Total volume, 5.0 ml. Incubation, one hour. Enzyme activity was expressed in terms of glucose liberated per dry matter on the basis of the same weight.

TABLE I

Effect of high temperature on β-glucosidase activity

Time for treatment	Relative activity (%)			
	40° C.	50° C.	60° C.	70° C.
5 min.	102.4	100.3	86.4	9.2
10	100.4	96.1	61.9	4.9
20	94.2	81.6	22.3	4.9
Control	100.0	100.0	100.0	100.0

for 5 to 20 minutes. At 40° C. no effect was observed with a 10-minute exposure and slight inhibition was recognized after exposure for 20 minutes. The treatment at 50° C. for 10 minutes resulted in a slight inhibition and that for 20 minutes in a 20 per cent inhibition. The treatment at 60° C., however, resulted in a markedly increasing loss of the activity according to the prolongation of exposing period up to 80 per cent of inhibition. By applying a high temperature of 70° C., most of the activity was lost within 5 minutes.

Inhibition by heavy metals

In insects the inhibition of β-glucosidase by heavy metals has been reported for the ventriculus of the adult cockroach (Newcomer, 1954). Inhibition of the enzyme solution obtained from the silkworm midgut with varying concentrations of $AgNO_3$ or $HgCl_2$ resulted in varying degrees of inhibition, as shown in Table II.

Effect of organic acids

Inhibition of β-glucosidase by organic acids has been reported for *Penicillium*, when phenyl β-glucoside was used as substrate (Murakami, 1950). Malic, fumaric, and citric acids were tested for their inhibitory effects on silkworm midgut β-glucosidase at a final concentration of 0.05 *M*. The results showed that no appreciable inhibition was observed, when salicin was used as substrate.

Effect of toluene

Newcomer (1954) has shown that an activation of β-glucosidase by toluene does not occur in the cockroach. The effect of toluene on midgut β-glucosidase in the silkworm was tested and no activation was recognized. Toluene was, there-

TABLE II

Inhibition of β-glucosidase activity by heavy metals

Final concentration (*M*)	Inhibition (%)	
	$AgNO_3$	$HgCl_2$
1×10^{-2}	95.3	—
2×10^{-3}	76.4	86.7
1×10^{-3}	57.9	52.8
2×10^{-4}	26.4	23.5
1×10^{-4}	1.1	15.5
0	0.0	0.0

fore, added to the incubation mixture when a long period of incubation was necessary.

Distribution of the activity in the midgut

The activity of β-glucosidase was compared among different parts of the midgut, *i.e.*, anterior, middle, and posterior midguts. The measurement of the activity was carried out with fresh homogenates and the results are shown in Figure 4. It is evident that the majority of the activity is concentrated in the posterior midgut, while a very low activity is found in the anterior and middle midguts.

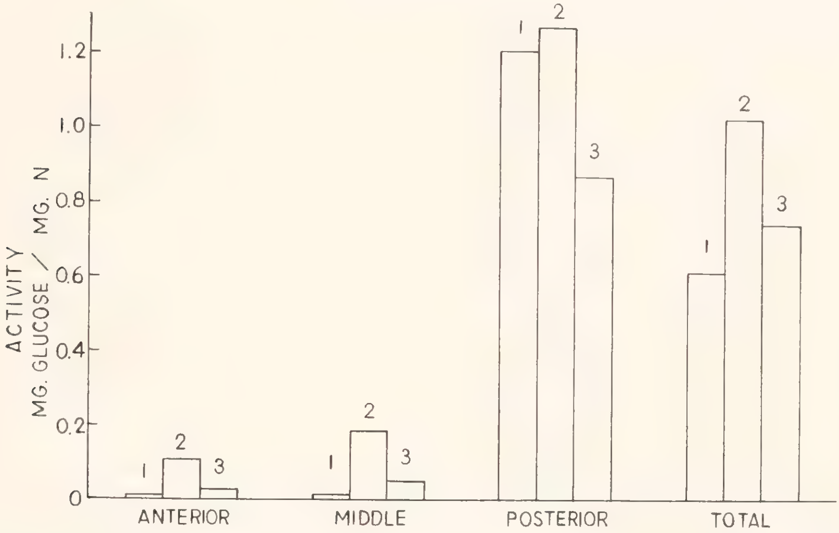


FIGURE 4. Distribution of β-glucosidase activity in the different parts of the midgut. 1, fourth day; 2, seventh day; 3, eighth day of fifth instar.

The change in the activity during larval development

The changes in β-glucosidase activity according to the development were measured with fresh midgut homogenates during fourth and fifth larval instars. The measurements were made with spring silkworms, the rearing temperature ranging approximately from 20 to 25° C., and with summer silkworms, the rearing temperature ranging approximately from 25 to 30° C. Though the activity expressed by unit glucose freed per nitrogen was higher in spring silkworms than in summer silkworms, the changes in the activity were almost the same in both (Fig. 5). In general, the activity was low during the fourth and early fifth instars. A marked increase in the activity occurred at the middle period of the fifth instar, and was maintained for a few days. Then the activity dropped suddenly and reached the lowest level during cocoon-spinning.

Precipitation by ammonium sulfate

In a preliminary experiment it was noticed that the majority of the activity was precipitated between 0.3 and 0.5 saturation with ammonium sulfate, when a suspen-

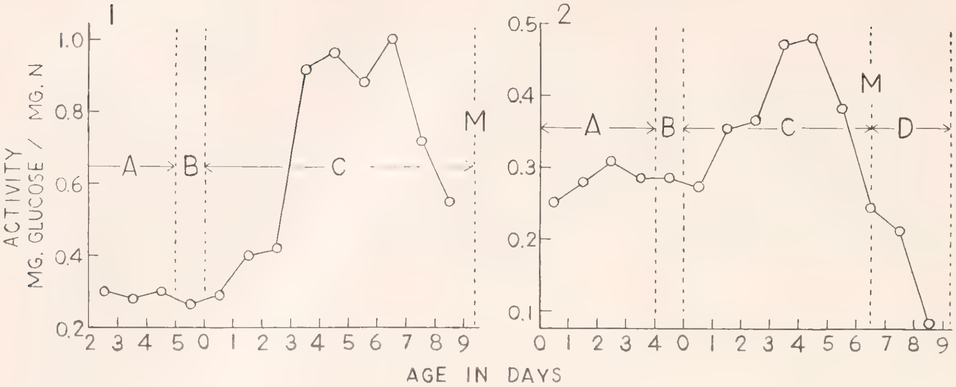


FIGURE 5. Change in β-glucosidase activity during larval development. *1,* spring rearing; *2,* summer rearing. Fresh homogenates were diluted to ½ in spring, and ¼ in summer. A, fourth instar; B, fourth molting period; C, fifth instar; D, cocoon-spinning period; M, maturity.

sion of acetone powder was used. Therefore, an attempt was made to purify β-glucosidase of the midgut by means of ammonium sulfate precipitation. Subsequently, the precipitation procedure was repeated several times by increasing the concentration of ammonium sulfate progressively. Table III shows one of the results obtained. Acetone powder made with posterior midguts was suspended in water in the cold for four hours; this suspension was used for the precipitation experiment. The specific activity of this suspension was 1.83 and that obtained with supernate after centrifugation at 12,000 × *g* for 10 minutes was increased almost three times, as seen in Table III. About 90 per cent of the original activity was found in the supernate. Until 0.350 saturation, very slight activity was precipitated. Most activity was precipitated between 0.350 and 0.450 saturation and the highest specific activity was obtained between 0.375 and 0.425 saturation. The specific activity was increased to about 4 times that of the supernate, and 10 times that of the original suspension. The application of ammonium sulfate precipitation thus seems to be to some extent useful for the purification of β-glucosidase.

TABLE III

Precipitation of β-glucosidase by ammonium sulfate

Saturation of ammonium sulfate	Total activity, mg. glucose	Specific activity, mg. glucose/mg. N	Recovery, %
Suspension	360.67	1.83	100.0
Supernate	327.88	4.37	90.91
0 –0.325	5.23	0.23	1.45
0.325–0.350	8.13	0.84	2.25
0.350–0.375	55.73	9.68	15.45
0.375–0.400	92.00	16.61	25.51
0.400–0.425	75.05	14.92	20.81
0.425–0.450	52.50	7.64	14.55
0.450–0.475	12.23	3.71	3.39
0.475–0.500	7.30	3.97	2.02

Acetone powder used in the present study was recognized to possess amylase and invertase, in addition to β-glucosidase. An attempt was therefore made to separate β-glucosidase from amylase or invertase by means of ammonium sulfate precipitation. The result showed that the precipitates at between 0.375 and 0.425 saturation contained all of three activities at almost the same level (β-glucosidase, 56.5%; amylase, 41.7%; invertase, 57.0%).

Several methods have been presented for the standardization of β-glucosidase (Veibel, 1950). An enzyme efficiency was obtained with a few fractions precipitated by ammonium sulfate by the use of phenyl β-glucoside as the substrate (final concentration, 0.052 *M*), according to the procedure by Helferich (1933, 1938). A high value of enzyme efficiency, 0.898, was obtained with the precipitate at between 0.375 and 0.425 ammonium sulfate saturation, while 0.170 with the precipitate between 0–0.375 saturation, 0.154 with that between 0.425–0.700 saturation, and 0.095 with the original supernate of acetone powder suspension.

Furthermore, β-glucosidase of the silkworm midgut was recognized to hydrolyze cellobiose as the substrate.

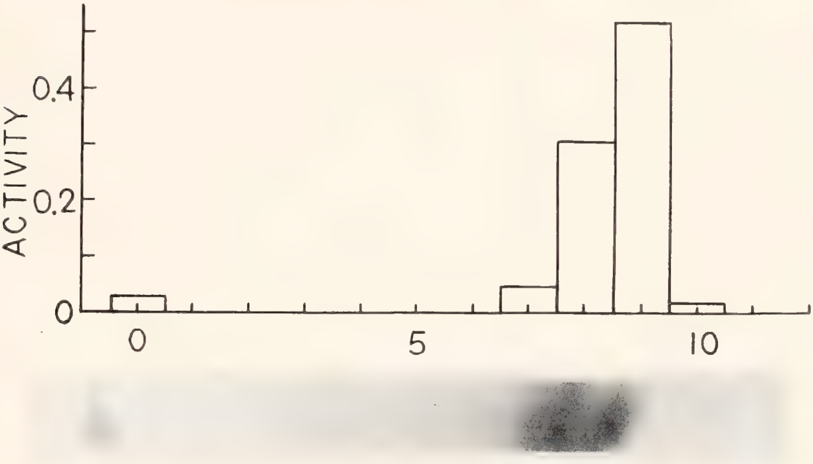


FIGURE 6. Distribution of β-glucosidase activity after paper electrophoresis. The activity was expressed in terms of mg. glucose liberated.

Purification by paper electrophoresis

Robinson (1956) has applied a paper electrophoretic procedure for the separation of β-glucosidase from β-glucuronidase in the locust-crop fluid. A similar procedure was also tested with β-glucosidase from the silkworm midgut. Either an acetone power supernate or the precipitate at 0.425-0.450 saturation of ammonium sulfate was used for the experiment. An enzyme fraction was subjected to electrophoresis on filter paper (Tôyô No. 51) 3 cm. × 30 cm. in 0.1 *M* phosphate buffer at pH 5.8, 150 volts, 5 ma., for 8 hours. Subsequently, the paper was cut into half along the long side of the paper. One of the divided strips was dried and suspended in the staining solution (Amido Black) for proteins, and the other was cut into one-cm. sections starting from the original spot, each section being chopped into small pieces which were immediately placed into a test tube with 1.0 ml. of phosphate buffer (pH 5.8). The test tube was kept at 5° C. for 5 hours to extract the enzyme, then heated to 37° C. with the addition of an appropriate amount of salicin solution (final concentration, 0.0125 *M*). After a 16-hour incubation, the amount of sugar liberated was determined. The results of the enzymic test, as well as of the staining test on the precipitate by ammonium sulfate, are shown in Figure 6, where the β-glucosidase activity is shown in the form of histograms. β-Glucosidase appeared in the locations corresponding to the staining test on the other strip. Little activity was found at the original point, where a protein band remained. However, when the whole midgut suspension was used, another two protein bands were recognized on the paper, which were considered to have been removed by the procedure of the precipitation with ammonium sulfate. The separation of invertase from β-glucosidase by electrophoresis was unsuccessful.

β-*Glucosidase activity in the midgut of jaundice-diseased larvae*

Two types of polyhedroses are known to occur in the silkworm, one of which is called cytoplasmic polyhedrosis, with the formation of the polyhedral bodies in the midgut cytoplasm. Several days after the infection, midgut tissue becomes white, which is a typical symptom of this disease. β-Glucosidase activity was compared between normal and infected larvae. The activity was always lower in diseased larvae reduced to 61 per cent of the normal larvae (incubation period, two hours) and 82 per cent (incubation period, 6 hours).

β-*Glucosidase activity in the digestive fluid*

The activity of the digestive fluid per unit nitrogen of early fourth instar larvae was one-third that of the midgut or less, while that of late fourth instar and fifth instar larvae was less than one-tenth that of the midgut. β-Glucosidase activity was also recognized in the digestive fluid of the amylase-free strain, which is deficient in amylase activity in the digestive fluid. The experiment performed at the same time showed that the midgut of the amylase-free strain possessed the same level of activity of β-glucosidase as the normal strain.

Discussion

The exact physiological role of β-glucosidase of the silkworm midgut in digestion is at present not well understood. The enzyme activity on the basis of the same unit is, however, higher in the midgut than in the digestive fluid. This seems to suggest that β-glucosidase in the midgut cells is of rather more importance than that in the digestive fluid. The optimal pH of midgut β-glucosidase ranges approximately 5.0 to 6.4, while an effort was unsuccessful to determine its range in the digestive fluid. A possible role of β-glucosidase in the cells of the midgut in digestion is also deduced from the fact that the pH value of the digestive fluid is strongly alkaline, as much as 10.0. The movement of food through the gut is generally fast in the silkworm larva, occurring within a few hours. Thus, even though the degree of participation of this enzyme in digestion as a whole is still unknown, the possibility remains that the mulberry carbohydrates which have not been completely hydrolyzed in the lumen of the gut might be hydrolyzed after absorption in the midgut tissue. It is interesting from the standpoint of comparative physiology that the intercellular enzyme might participate in the digestion. Although conclusions drawn from a study of enzyme alone are generally open to question in regard to the physiological role in intact organs, a good correlation was found between pure compounds supporting growth and the presence of digestive enzymes in insects (Day and Waterhouse, 1953). Koike (1954) could not demonstrate cellulase in the digestive tract of the silkworm and Hiratsuka (1917) has shown that cellulose is not utilized by silkworm larvae. This is the same situation as reported for the hepatopancreas of *Porcellio* (Newcomer, 1956) where an activity of β-glucosidase was demonstrated without that of cellulase. β-Glucosidase of the midgut or of the digestive fluid of the silkworm seems to hydrolyze β-glucosides contained in the mulberry leaves. A few papers have been so far published on glucosidic compounds in the mulberry leaves; recently Hamamura and Naito (1956) isolated arginine β-glucoside and the presence of glucosides of the pigment has also been reported (Oshima and Nakabayashi, 1951). There is no doubt that these glucosides and possibly other not yet identified glucosides are utilized by the larvae.

The results on the characterization experiments suggest that the β-glucosidase of the midgut is very much similar to that in plants (Veibel, 1950). The enzyme efficiency of β-glucosidase of the midgut is rather higher than that obtained with plants (Pigman, 1946).

A variation in the digestive enzyme activities of different parts of the midgut, as well as in the ability of the absorption of the nutrients, is well known in insects (Day and Waterhouse, 1953; Waterhouse and Day, 1953). The physiological or digestive differentiation in the different portions of the midgut of the silkworm is still not well known in many respects. However, the highest activity of β-glucosidase was found in the posterior midgut (Fig. 4). Matsumura and Oka (1935) have shown that the activity of amylase or invertase is also the highest in the posterior midgut. The glycogen content is increased most markedly in the posterior midgut after sugar ingestion (Horie and Tanaka, 1957) and the highest phosphorus metabolism was obtained also in this portion (Ito, Horie and Tanaka, in press).

The authors wish to express their thanks to Prof. G. S. Fraenkel of the University of Illinois for reading the manuscript.

SUMMARY

1. The presence of a β-glucosidase was demonstrated in the midgut of the silkworm larva, *Bombyx mori*.

2. The enzyme has a pH optimum of approximately 5.2–6.2 and the *Km* value was 0.013 with salicin as a substrate.

3. The action of the enzyme was slightly inhibited at a temperature of 40° C., and strongly inhibited at 70° C. An inhibition by silver or mercury salts was also observed, while no inhibition was found by organic acids. No activation by toluene was demonstrated.

4. Most of the activity in the midgut was concentrated in the posterior portion.

5. The enzyme activity varies according to larval growth, being lower at the beginning of the fifth instar, higher after the middle of the instar, and again lower during cocoon-spinning.

6. The enzyme activity was concentrated 10 times by means of ammonium sulfate precipitation at a saturation of 0.375–0.425. Separation by the paper electrophoretic method was successfully applied for this fraction, but it was unsuccessful for separating β-glucosidase from other enzymes.

7. Virus-infected larvae showed a decrease in enzyme activity, compared with normal larvae.

8. β-Glucosidase activity in the digestive fluid was much lower than that in the midgut. A mutant, amylase-free strain possessed in the digestive fluid the same level of β-glucosidase activity as the normal one.

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THE EFFECTS OF THIOUREA AND SOME RELATED COMPOUNDS ON REGENERATION IN PLANARIANS[1]

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During recent years much research has been devoted to the effects of the administration of various anti-thyroid agents to vertebrate animals. Interest is due to the fact that these agents have been demonstrated to inhibit the activity of the thyroid gland. Only a few studies have been made of the effects of such drugs on invertebrates, and the majority of these deal with the effects of the goitrogens on fertilized eggs and developing embryos. Bevelander (1946), using fertilized sea urchin eggs placed in test solutions of 0.1–1.0% thiourea in sea water, found that at a concentration of 1.0% no cleavage occurred, but cleavage was normal in a similar concentration of urea, indicating the inhibition of cleavage was not due to any osmotic effect. Lower concentrations produced a retardation in over-all growth rate. Rulon (1950), studying the modifications in developmental patterns in the sand dollar by thiourea, reports substantially similar results.

The present investigation was undertaken in order to study some comparative effects of varying concentrations of thiourea and related compounds on an invertebrate beyond the embryonic stage. For this study a species of planarian, a fresh-water flatworm, was chosen. In this animal, when the tail is separated from the body by a dorso-ventral cut posterior to the pharynx, the body will produce a new tail, and the separated tail will regenerate all missing structures, becoming a new and independent organism. A study was made of the rate of growth of a new tail by the body, and of the time required for the appearance and development of the regenerated organs in the newly formed worm. Observations were also made of any modifications in the regenerating structures, due to the action of the goitrogens, and of pigment loss or lack of development, both in the new tissue and in the old, mature cells.

MATERIALS AND METHODS

The animals used in this study were specimens of *Dugesia tigrina*, collected in a stream near Baltimore, Maryland. Stock animals were fed once a week. Experimental animals were taken five days after feeding, and were not fed during the experiment.

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[1] A contribution from the Department of Biology, The Catholic University of America, Washington, D. C. This paper is based on the author's dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science.

The writer wishes to express her appreciation to Dr. E. G. S. Baker, major professor, now Chairman of the Department of Biology, Drew University, Madison, New Jersey, and to Dr. W. G. Lynn, Professor of Zoology, of The Catholic University of America, for their many helpful suggestions during the course of the investigation.

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Three chemicals were used in the study: thiourea, phenylthiourea, and thiouracil. Since a comparative study of the effects was to be made, three series of experiments were performed, using concentrations of 0.005%, 0.01%, and 0.02% of each chemical. Worms chosen for experimentation were as near the same size as possible, varying from seven to nine millimeters in length. Tails were severed a short distance behind the pharynx, and placed in fingerbowls of the proper solution. These were stacked to prevent evaporation. The bodies were placed similarly in other fingerbowls. The worms were handled with sable-hair brushes, or wide-tipped medicine droppers. Control animals were kept in tap water.

In Series I, the experimental animals were placed in 0.005% solutions of the chemicals. No observations were made on the regenerating tails the first day after cutting. Beginning with the second day, the tails were observed every day for ten days, then on the fourteenth, eighteenth, and twenty-fifth days. At the end of seven days, the worms in each chemical were divided into two groups. One group was kept in the chemical until the end of the experiment; the other group was returned to water to see if any of the effects noted were reversible.

For observation, the tails were placed in a drop of the solution on a microscope slide, and observed through the low-power objective of a compound microscope, using a blue filter in a standard lamp. Information was obtained concerning the time in days required for healing to take place, and for eyes, proboscis, and sense lobes to form. Observations were also made concerning the color and appearance of the eyes and of the proboscis, and of such noticeable special effects as might occur.

The bodies of the worms were observed every second day for the first week, and every third day thereafter. At the end of seven days the worms in each chemical were divided into two groups. One group was returned to water; the other remained exposed to the goitrogen. The rate of regeneration was observed by measuring the lengths of the worms on successive days. The effect of the chemicals on the pigmentation was noted.

For measuring, a somewhat modified form of the method originated by Wulzen (1927) was employed, and the average length of worms in each solution was computed. Graphs of growth rate were made, plotting average lengths, calculated to 0.1 mm., against time in days. In order that a better comparison of growth rates in the different solutions and series might be made, the daily average length in each group was recalculated, using as the original average length on the day of cutting that average exhibited by the water controls, namely, 5.6 units.

A second series of experiments, using a concentration of 0.01% of each of the chemicals, was performed. No other change was made in either method or materials. A third series, using a 0.02% concentration was likewise performed, but due to the toxicity of phenylthiourea at this concentration, a comparative study of effects at correspondingly higher concentrations was not attempted.

The Effect of the Goitrogens on Severed Tails

Healing. In the normal planarian, when a tail is severed, the cut edge contracts, forming a pronounced, black indentation, semi-circular in shape. Within two to three days, as healing progresses, relaxation occurs, and the newly forming, unpigmented flesh is protruded forward in a more or less triangular shape as the worm glides about. In the worms treated with chemicals, the healing process was noticeably slowed. When thiourea was used, the effect appeared to be in proportion to the concentration used. Worms placed in a 0.005% solution were all healed on the third day, in a 0.01% solution on the fourth day, and in a 0.02% solution on the fifth day.

The phenylthiourea was markedly more effective than the thiourea, even in the lower concentrations. It was not until the sixth day that healing occurred in all worms placed in a 0.005% solution, and in a 0.02% solution complete healing did not occur. The effect of the thiouracil solutions on healing, while greater than that of the thiourea, was less than that of the phenylthiourea. Worms placed in a 0.005% and in a 0.01% solution were healed by the third day, but six days were required for complete healing of those placed in the 0.02% solution.

Formation of sense lobes. When a head is forming in a regenerating planarian, by the fourth or fifth day the triangular protuberance of unpigmented new flesh has become sufficiently large that the animal, in moving about, exhibits the beginnings of sense lobes by protruding and withdrawing, seemingly at will, a small bit of tissue on either side, just anterior to the healed cut. In this experiment, it was found that the 0.005% solution of each of the three chemicals and the 0.01% concentration of thiourea and thiouracil were ineffective in retarding this. All the animals in these solutions were able to produce sense lobes by the fifth day.

The other concentrations used were more effective in this respect. Sense lobes appeared in all worms in the 0.02% solutions of thiourea and thiouracil on the sixth day, and in the 0.01% concentration of phenylthiourea on the eighth day. It was not until the tenth day, however, that the worms in 0.02% phenylthiourea showed this stage of development. In the worms returned to water from higher concentrations of the chemicals, the sense lobes appeared within twenty-four hours after return, or by the eighth day.

Proboscis development. The first definite sign of a developing proboscis in a severed tail can be seen in a freely moving planarian on the third or fourth day after cutting. A smooth, tan-colored protuberance appears at the point where the two sides of the digestive tract have grown together, and grows caudally until its length is about four times its width. Pigmentation and wrinkling, the latter due to an increase in real but not apparent length, occur on the fifth or sixth day after cutting, in the normal worm.

In this experiment both the 0.005% and the 0.01% solution of each of the three chemicals had little effect on the time required for the appearance of the proboscis, or on its subsequent development, but each of the chemicals was effective at a concentration of 0.02%. At this concentration the organ could be seen in all the animals in thiourea and thiouracil on the fourth day, but it was not until the fifth day that it could be found in all of the worms in phenylthiourea. Further development of the proboscis was also affected. By the fourteenth day the worms in both thiourea and phenylthiourea exhibited a very immature proboscis, shorter and narrower than is normally found on the fourth day. The latter solution was particularly toxic. The animals in thiouracil fared better. In them the proboscis, while less mature in appearance than those in the water controls, was apparently able to function normally. The effect was reversible in the worms returned to water at the end of seven days. In these worms, by the fourteenth day the proboscis was as developed, pigmented, and wrinkled as those of the water controls.

Eye formation. Eye formation in the normally regenerating planarian begins quite early. By the third day definite, tiny, black eyespots can be seen under the low power of the microscope, and by the sixth day the spots have become large and black, curved and smooth in outline on the median side, and concave and slightly granular on the lateral side.

In this experiment the effect of the thiourea was quite varied as far as individual worms were concerned, but the concentration did not seem to cause a marked difference. At all three concentrations the developing eyes were somewhat smaller and more granular in appearance than those of the water controls. The black pigment that formed began to disappear irregularly on the sixth day in Series I and II, and on the fifth day in Series III. On the seventh day, before the transference of half the animals to water was made, it could be seen the pigment was disappearing to a greater or lesser extent in the eyes of all the worms at all three concentrations. During the following week a change could be noted daily. All the worms which were kept in the 0.005% solution of thiourea lost all eye-pigment by the eighteenth day. The animals in the 0.02% solution of thiourea lost all eye-pigment by the tenth day of subjection to the chemical, but in each one there persisted a distinct, ghost-like outline of the eye shape, very faintly yellowish-pink in color. The 0.01% concentration was variable in its effects. By the twenty-fifth day, in one of the worms there was a nearly normal amount of black pigment, while in the others the pigment was nearly gone, but in no case was it completely absent. In contrast, the worms which were returned to water gained pigment little by little, until by the fourteenth day they closely resembled the water controls.

The phenylthiourea, at all concentrations used, inhibited pigment formation completely in the developing eyes, although the eyes themselves could be seen in faint, ghost-like outline, faintly yellowish-pink in color. In Series I, the eyes of the worms which remained in the chemical showed during the second week a faintly brown, smooth outline. By the eighteenth day this was more pronounced, and by the twenty-fifth day reddish-tan granules had begun to appear in the eyes. It is possible that black pigment might have eventually developed, but the regenerating tails, which had been without food over three weeks, had become so small that sustenance was necessary for their continued existence, and the experiment was brought to a finish.

In both the two higher concentrations of phenylthiourea, the worms which remained in the chemical during the entire experiment showed practically the same effect. After the eye outlines appeared, there was no change until the fourteenth day, when a slightly pinker color began to show. In the worms in Series II, the eyes were full size and very pink in color on the twenty-fifth day, but the worms in Series III had died and disintegrated by the eighteenth day, so that further observation was impossible.

In the worms which were returned to water from each of the three concentrations of phenylthiourea, a steady development of pigment followed. The smooth outline became darker and a golden-brown color developed inside. This gradually changed to a reddish-brown, then black. The eye outlines became granular as the darker colors appeared. By the fourteenth day, the eyes of all returned to water appeared like the eyes of the water controls, with the exception that these retained a slightly reddish cast. By the twenty-fifth day these were indistinguishable from the water controls.

Solutions of thiouracil showed much less effect than solutions of either thiourea or phenylthiourea. In all cases the general effect of the chemical was to cause the eyes to become slightly more granular in appearance than is normal, and to become slightly reddish in spots as the pigment partially disappeared. This was more pronounced in the higher concentrations, but in no case did the pigment completely disappear, even after twenty-five days exposure to the chemical. Worms returned to water on the seventh day regained normal eye appearance within three days.

Skin pigmentation. During all series careful attention was given to possible effects of the chemicals on skin pigmentation, both in mature cells and in newly forming tissue. No bleaching effect was noticed under the influence of any one of the three chemicals, at any concentration used, up to twenty-five days, when the experiment was terminated.

THE EFFECT OF THE GOITROGENS ON GROWTH RATE

By a comparison of the average lengths of the worms, as measured on succeeding days, it was found that regenerating planarians in water, at a controlled temperature, exhibit a characteristic growth curve. For the first four days after the tails are severed, rapid growth of the bodies occurs, followed by two days of slower growth. The maximum length is reached on the sixth day. Following this, if food is not given the animal, it must begin to live on its own tissues, and a decrease in length results. After a four- to five-day interval, the graph line begins to level off somewhat. Another period of rapid decline follows, then another period of levelling-off.

The characteristic growth curve of planarians in water is shown in Figure 1, together with a typical response of the animals to the effects of the goitrogens. In this graph, the regenerative growth rate of worms subjected to a 0.02% solution of thiourea, and of those returned to water at the end of seven days, is compared with the curve exhibited by the water controls. It will be noticed the peak of growth occurs on the sixth day for both groups of animals, although the peak attained by the experimentals is lower. The graph line for the planarians returned to water shows the characteristic lessening of retardation of growth. A study of the comparative effects of thiourea at different concentrations reveals that the 0.005% concentration is least effective in depressing the growth rate, and recovery from exposure to it follows most rapidly; the 0.01% solution is most effective in depressing the growth rate during the first few days of exposure; and the 0.02% concentration, while not most effective in depressing the initial growth rate, is much more potent after long exposure.

The distinct lessening of retardation of growth in animals returned to water at the end of seven days was quite apparent in all three series with each chemical used. In the majority of cases the lessening of effect was so pronounced that a second growth peak was reached. This was especially noticeable in animals exposed to thiouracil. In Figure 2 the second growth peak is shown to have occurred on the fourteenth day, or seven days after the planarians were returned to water from 0.02% thiouracil. The occurrence of the second growth peaks ranged from the eleventh to the fourteenth day.

It was found by a comparison of the effects produced by each of the goitrogens at a concentration of 0.005% that the thiourea affected the rate of growth less at this concentration than did either thiouracil or phenylthiourea, and that the latter was the most effective. This conforms with the findings above of the influence of the chemicals on the regeneration of missing organs in severed tails.

A study of the growth rate of planarians in a 0.01% solution of the chemicals showed that, while initial exposure to thiourea at this concentration was not highly effective, continued exposure produced a marked retardation in growth, and a return to water allowed nearly normal growth to be resumed. At this concentration both phenylthiourea and thiouracil were found to be quite effective in depressing initial growth, so much so that the peak of growth was not only quite low, but was reached seven to eight days after exposure to the chemical, or one to two days later than the peak observed in the water controls.

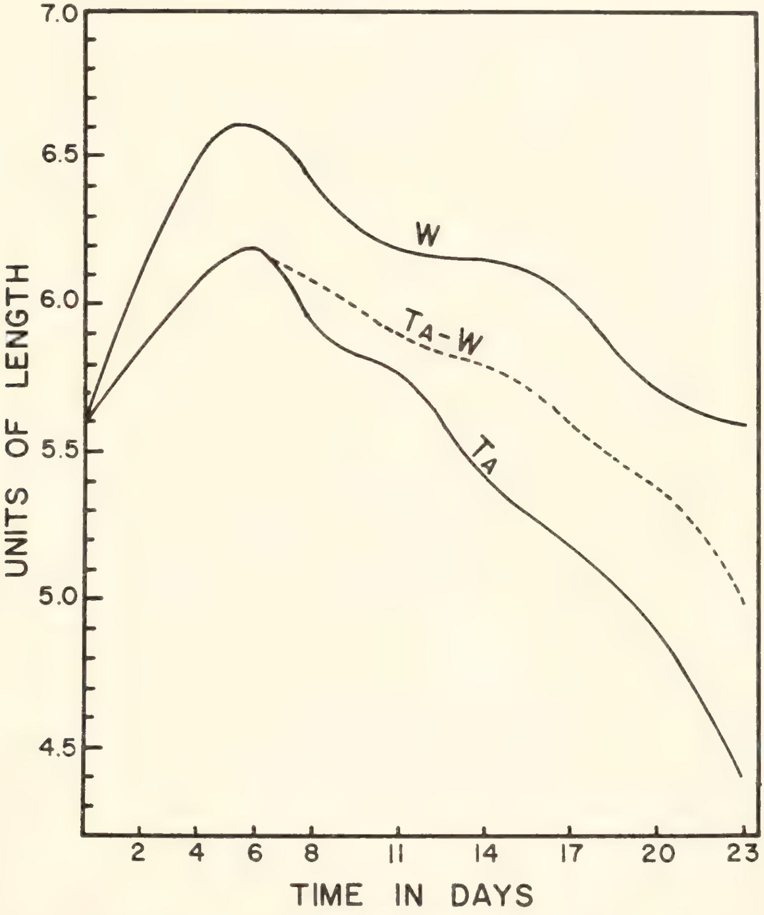

FIGURE 1. Growth rate curve of planarians exposed to 0.02% thiourea (Ta) and of those returned to water at the end of seven days (Ta-W) compared with the characteristic curve of water controls (W).

A comparison of the effects of exposing the experimental animals to a 0.02%

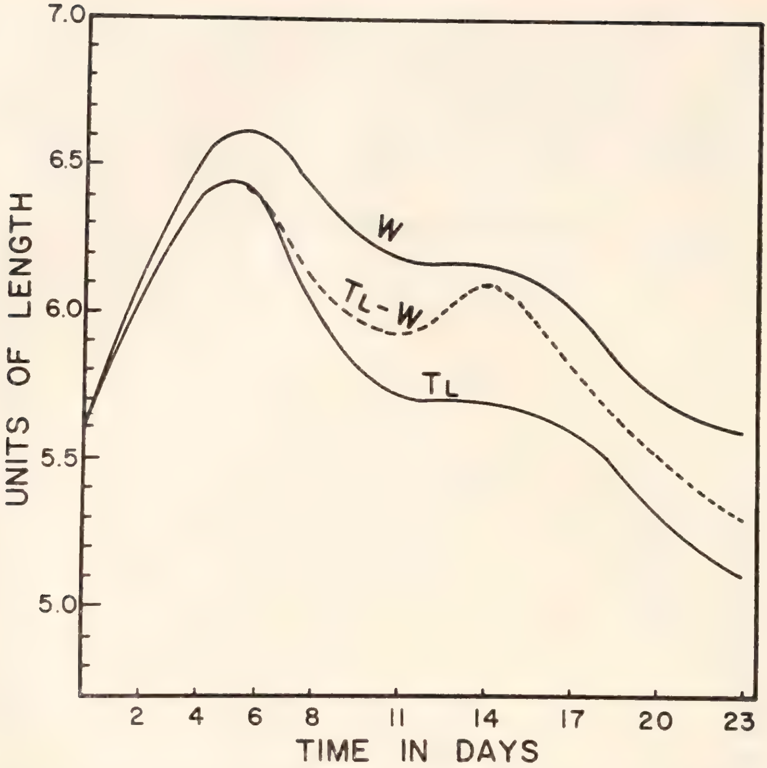

FIGURE 2. Growth rate curve of planarians exposed to 0.02% thiouracil (TL) and of those returned to water at the end of seven days (TL-W) compared with the characteristic curve of water controls (W).

concentration of the chemicals revealed that, at this concentration, thiouracil was least effective in retarding growth, while phenylthiourea was most effective. A marked depression, approaching toxicity, followed continued exposure to both thiourea and phenylthiourea, but a noticeable recovery was made when the animals were returned to water after a week's exposure. This, too, is in accord with the facts noted above.

DISCUSSION

In the present study, it was found that the normal regenerative powers of the planarians were reduced by the administration of goitrogenic agents in varying concentrations, and that the effect was more pronounced as the concentration was increased. This is in conformity with the findings of Lynn (1948) and Rulon (1950). Lynn, testing two of the thioureas on a toad, *Eleutherodactylus ricordii*, which possesses no aquatic larval stage, found that a concentration of 0.001% thiourea was ineffective, a concentration of 0.005% was slightly effective, and that a concentration of 0.05% thiourea caused a definite retardation in development. Rulon reported that continuous exposure of newly fertilized eggs of *Dendraster* to low concentrations of thiourea resulted in slight inhibition of development, and that with higher concentrations the degree of inhibition increased.

In this experiment the depression of growth rate by the goitrogens, noticeable to some extent at all concentrations, was shown not only by the lower peak of growth as exhibited by the graphs, but also by the fact that certain of the concentrations slowed the initial growth sufficiently that the peak was reached after seven to eight days' exposure to the chemical, at a time when the period of rapid decline was apparent in the water controls. A possible explanation of this is that the lowered metabolic rate allowed a longer use of the food present in the animal, before the necessity of subsisting on its own tissues became imperative.

The second growth peaks noted in the majority of animals returned to water, which occurred at a time when a levelling-off period was to be found in the water controls, were apparently due to an upsurge of metabolic activity following the release of the animals from the influence of the goitrogens. This effect appears to be similar to that noted in the severed tails, when rapid reconstitution of deficient organs followed the return of the animals to water.

None of the chemicals used had any appreciable effect on head formation, the appearance of functioning sense lobes, or the development of the proboscis, when used at a concentration of 0.005%, and only phenylthiourea exhibited a marked modifying action at a concentration of 0.01%. All three chemicals, at a concentration of 0.02%, produced a distinct retardation in all phases of organ development. The results of this study show that not only is the retardation of the metabolic rate of planarians, as evidenced by the rate of regeneration, influenced by the degree of concentration to which the animals are subjected, but that certain goitrogens are more effective than others in this respect. In all phases of the study, phenylthiourea was found to be more potent in repressing the rate of regeneration, and in causing modifications in developing organs, than either thiourea or thiouracil. This, too, is in agreement with the results obtained by Lynn (1948), who found that a 0.005% concentration of phenylthiourea was as effective in retarding embryonic development as was the 0.05% thiourea.

Reports of several workers indicate that the development of pigmentation in the animal body is intimately associated with the metabolic process. Lynn (1948), treating leptodactylid embryos with 0.005% phenylthiourea, found that not only was there a definite retardation in development, but that within three days the experimentals were noticeably lighter than the controls, and by the sixth day all visible dark pigment, both in the skin and in the retina of the eye, had disappeared. Frieders (1954), studying the effect of the same chemical on fish, found that the animals showed a definite loss of body pigment, and that a gradual but noticeable loss of pigment could be observed in the eyes. At the same time, the growth rate of the experimentals was much slower than that of the controls.

While no bleaching effect in regard to skin pigmentation was noted at any time in this experiment, it was found that all three chemicals interfered to some extent with the production of eye-pigment at all concentrations, the effect increasing as the concentration was increased. That the goitrogens inhibited pigment formation, not the development of the eye itself, was shown by the fact that the planarians, particularly those in phenylthiourea, developed eye outlines, although pigment did not appear.

In this study, as in those cited above, the rate of metabolism of the planarians, as evidenced by the growth rate and by the appearance of new organs appeared to parallel the speed or slowness of pigment formation. It is probable that a fundamental correlation exists between the production of animal pigment and the production of chemicals which exert a controlling influence on the metabolic rate. The fact that goitrogens affect metabolism and pigment formation similarly in both vertebrates and invertebrates lends support to this view.

SUMMARY

1. A study was made of the effects of the three goitrogens, thiourea, phenylthiourea, and thiouracil, on *Dugesia tigrina*, a species of planarian. Observations were made of the effects of the drugs on healing, head formation, proboscis development, eye and skin pigmentation, and regenerative growth rate.

2. Phenylthiourea was found to be most effective in preventing healing. Both thiourea and thiouracil retarded the rate of healing.

3. Higher concentrations of all three goitrogens were effective in retarding or suppressing the normal development of sense lobes and proboscis. Phenylthiourea was most potent. Lower concentrations were ineffective. The effect was reversible.

4. Phenylthiourea inhibited eye-pigment formation, but not eye formation. The effect was reversible. Thiouracil had little effect on the formation of eye-pigment. The effect of thiourea was varied.

5. Bodies with severed tails, placed in water, showed a characteristic growth curve when body length was plotted against time in days. Plotted curves of planarians in goitrogens, compared with controls, showed retardation of growth. Noticeable recovery was made upon the return of the experimentals to water.

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ALMYRACUMA PROXIMOCULI GEN. ET SP. NOV. (CRUSTACEA, CUMACEA) FROM BRACKISH WATER OF CAPE COD, MASSACHUSETTS

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An interesting cumacean was collected by W. D. Burbanck in the Pocasset River, Cape Cod, Massachusetts. Specimens were sent to Dr. Thomas E. Bowman at the Smithsonian Institution, who forwarded them to N. S. Jones for identification. In the following account N. S. Jones is responsible for the description and systematic remarks and W. D. Burbanck for the sections on habitat and general ecology.

GENUS ALMYRACUMA GEN. N.

Anterolateral angles of the carapace not developed. Second antenna of the male rudimentary, one-jointed, resembling that of the female. Second maxilla with two endites. First maxilliped with four joints, the last very small, and the epipodite with only rudimentary branchiae. Third maxilliped pediform, with an exopodite. Only the first and second peraeopods bear an exopodite in either sex.

ALMYRACUMA PROXIMOCULI SP. N.

Material examined. Pocasset River, Cape Cod, Massachusetts; W. D. Burbanck, collector; 15 March 1958; 11 males, 38 females (12 ovigerous), 8 juveniles.

Description. Ovigerous female. Length range from 3.2 to 3.7 mm. Integument thin, finely granulated, with a few scattered hairs. Color yellowish white with dark brown pigment spots specially concentrated on the lower part of the carapace and at the sides of the free thoracic somites. Eyes black.

Carapace two-sevenths of total body length, about as high as it is long, and slightly longer than its greatest width posteriorly; from the side the dorsal outline swells upwards behind the eyelobe and is further elevated at the posterior end; a dorsal groove is present between the branchial regions; a prominence is set on each side of the hinder end with a hollow running forwards from below it towards the eyelobe; the pseudorostrum is short with the lobes divided for about half their length above; the anterolateral margin is only slightly concave and without any angle. Eyes well developed with corneal lenses, set close together but distinctly separated forming a double eyelobe.

Five free thoracic somites clearly visible from above. Brood pouch containing 10–14 ova. Pleon somites smooth, the fifth the longest. Telsonic somite little produced posteriorly.

First antenna with the three joints of the peduncle not very different in length, the third joint slightly the shorter; the flagellum with three joints, the short third

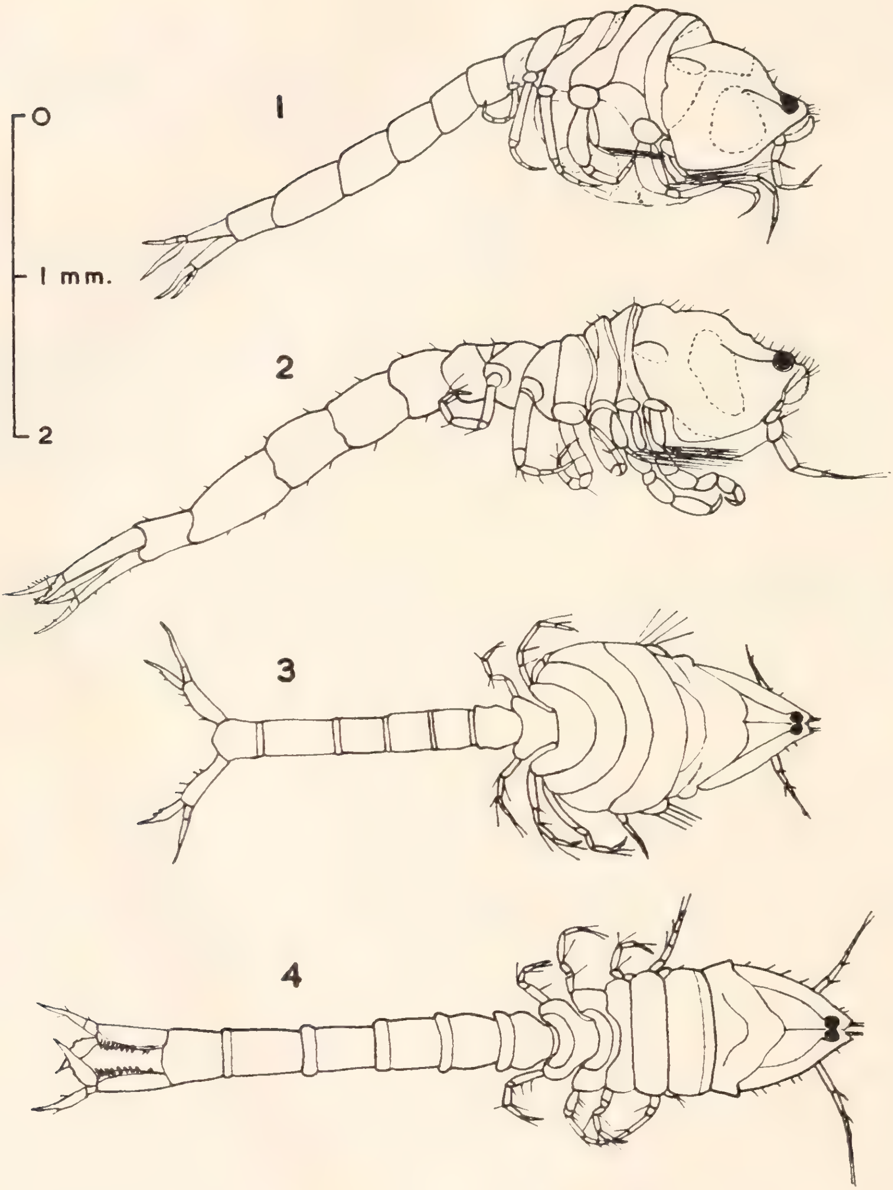

FIGURE 1. Paratype ovigerous female from side.
FIGURE 2. Paratype adult male from side.
FIGURE 3. Female from above.
FIGURE 4. Male from above.

joint carrying two aesthetascs; the accessory flagellum very small, one-jointed. Second antenna rudimentary, one-jointed, bearing two small plumose setae at its end.

Mandibles of normal shape, with molar process not styliform. First maxilla with two processes on the palp. Second maxilla normal with two upper lobes.

First maxillipeds with a lamellar merocarpus and a small end joint as in *Campylaspis;* the merocarpus bears a few flattened bifid spines as well as a number of pointed plumose spines; only two rudimentary branchial lobes are present on the epipodite. Second maxillipeds six-jointed with the basis curved outwards. Third maxillipeds pediform, bearing an exopodite; the basis less than half the length of the whole appendage, with its distal end not produced; the ischium short; the merus and carpus about equal in length and rather shorter than the propodus; the dactylus shorter than the propodus, ending in a fairly strong spine.

First peraeopods longer and more slender than the third maxillipeds; the basis stout, a little more than one-third the length of the whole appendage; the ischium fairly short; the remaining joints successively a little longer; the dactylus ending in a long slender spine. Second peraeopods much shorter than the first pair, with exopodite; the basis stout, as long as the next four joints together; the dactylus about twice as long as the propodus. Third to fifth peraeopods without exopodites; the third and fourth pairs with the basis slender, about as long as the remaining joints together; the fifth pair with the basis relatively shorter.

The uropods as long as the fifth abdominal and telsonic somites together; the peduncle fairly stout, about the same length as the subequal rami, with three or four setae on the inner edge; the outer ramus two-jointed; the first joint about one-fourth the length of the second; the second joint with a stout terminal spine and two setae on the outer and one on the inner edge; the inner ramus one-jointed with two strong spines on the inner edge.

Adult male. Length 3.8–4.3 mm. Carapace one-fourth of the total length of the body. The pleon relatively longer than in the female and more stoutly built. The dorsal outline of the carapace rather less elevated than in the ovigerous female and the lateral protuberances more prominent. Rather more scattered hairs are present.

The appendages are similar to those of the female except as follows: third maxillipeds with all the joints stouter; first peraeopods much more stoutly built, and with the dactylus less than two-thirds the length of the propodus and its terminal spine short and stout; the peduncle of the uropods relatively stouter and longer than in the female, about 1½ the length of the rami, with several basal setae and 10–12 stout spines on the inner edge; the outer ramus similar to that of the female but more robust; the inner ramus broad in the basal half, with 6–8 strong spines on the inner edge and two setae on the outer edge, and with a subterminal plumose spinule.

The bases of the appendages bearing exopodites are not specially widened, and it may be noted that the second antennae resemble those of the female, being similarly rudimentary without any trace of a flagellum.

Holotype and paratypes. USNM No. 102259–102261.

Systematic remarks. A. proximoculi clearly must be placed in the family Nannastacidae for the following reasons: it has no separate telson; there are three

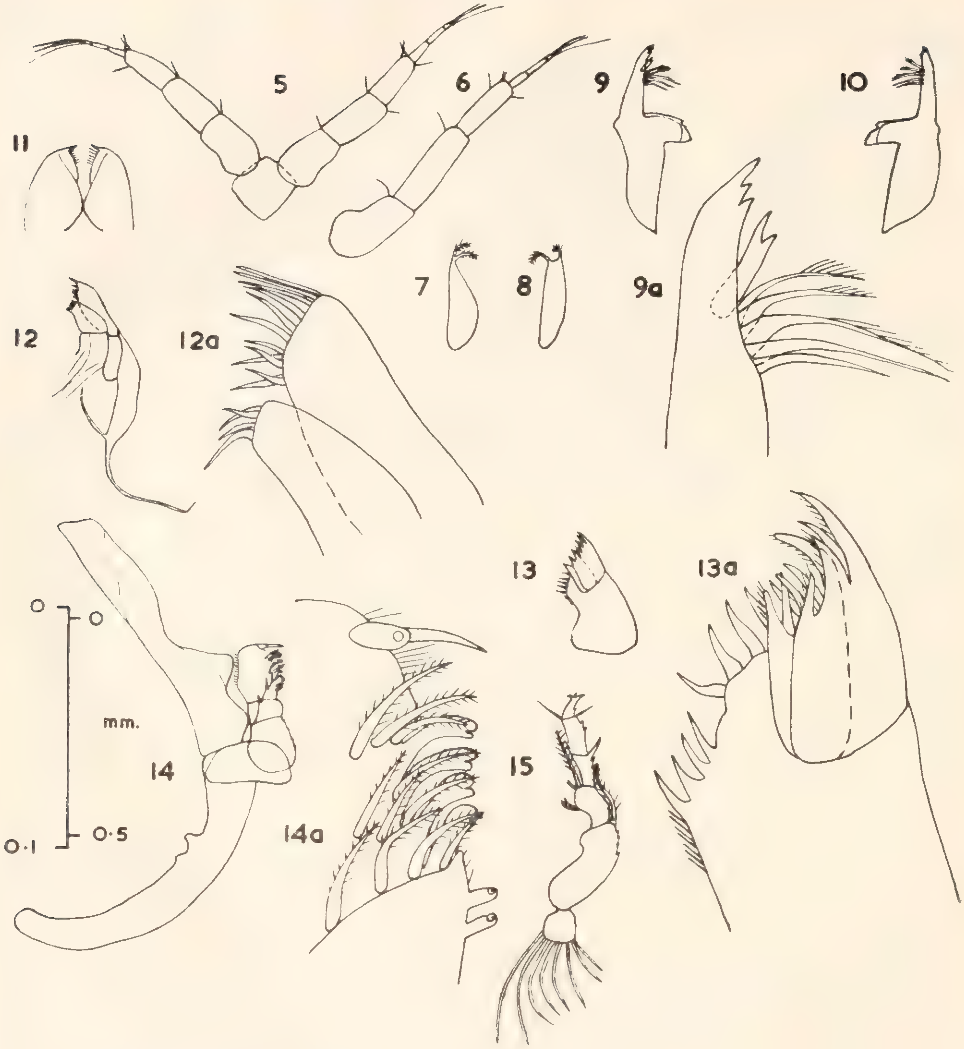

FIGURE 5. Female first antenna.
FIGURE 6. Male first antenna.
FIGURE 7. Female second antenna.
FIGURE 8. Male second antenna.
FIGURE 9. Female left mandible. FIGURE 9a. Same, distal and further enlarged.
FIGURE 10. Female right mandible.
FIGURE 11. Female labium.
FIGURE 12. Female first maxilla. FIGURE 12a. Same, distal end further enlarged.
FIGURE 13. Female second maxilla. FIGURE 13a. Same, distal end further enlarged.
FIGURE 14. Female first maxilliped. FIGURE 14a. Same, and joints further enlarged.
FIGURE 15. Female second maxilliped.

pairs of thoracic exopodites in the female; the male has no pleopods; the inner ramus of the uropods is one-jointed. It differs from all other described species of Cumacea in the rudimentary state of the male second antenna. There seems to be no doubt that the males are fully adult. They are larger than the females in the collection. They differ from the females in the shape of the carapace and the spinulation of the uropods. Ovigerous females were present and some of the males when captured were clasping females. There is some tendency towards reduction of the second antennae in certain species such as those of the genus *Lamprops*, where these appendages are used to clasp the female, and it is possible that in this species, where the second antennae are rudimentary, the greater development of the third maxillipeds and first peraeopods in the male is an adaptation for this purpose.

The male resembles the female and differs from most other members of the Nannastacidae in possessing a similar number of thoracic exopodites. *Picrocuma poecilota* Hale (1936), placed in the family Nannastacidae (Hale, 1945), has exopodites on the third maxillipeds and the first to third peraeopods in both sexes. It also resembles *A. proximoculi* in the absence of an anterolateral angle on the carapace, in the positioning of the eyes, and in the pediform shape of the third maxillipeds. The second antenna of the male has a reduced prehensile flagellum as in *Lamprops*. It differs considerably in other respects, however, such as the shape of the first antennae, mandibles and uropods.

Almyracuma proximoculi shows a combination of characters which excludes it from any previously defined genus of the Nannastacidae. The mouthparts on the whole resemble those of *Cumella*, with the exception of the first maxillipeds which are somewhat similar to those of *Campylaspis* but have only rudimentary branchial lobes. Its affinities are obscure but it is possibly closer to *Picrocuma* than to any other genus described at present.

Habitat. The type locality of the new cumacean is approximately one mile from the mouth of the Pocasset River, Cape Cod, Massachusetts (also known as Barlow's River) and nearly 100 yards downstream from a dam which separates the brackish part of the river from the last of a series of six confluent ponds (Fig. 26). A constant flow of fresh water from the ponds is appreciably augmented by cold water issuing from numerous springs lying in a semi-circle around the small flat where the cumaceans live.

At low tide the flat may be almost out of water (Fig. 27). Water running over parts of it has a pH of 6.0 and readings taken *in situ*, where cumaceans were living, ran as low as 4.4. The cumaceans live in a substrate of detritus and algae. At low tide the water in which they live has a salinity of less than 1 ‰. At high tide they are overlain by 3–4 feet of water which has a pH of approximately 8.0 and a salinity of about 30‰.

During the course of the fall, winter, spring and summer of 1957–58 the temperature of the substrate ranged from 3–20° C., with water temperatures slightly higher, 3–22° C. Less than 30 yards beyond where the cumaceans were living, Pocasset River froze over during the months January and February of 1958. No cumaceans were found in this area later in the year while they were present in the open areas during the coldest months, with one pair being found clasping in February. Most of the animals apparently breed during the month of March although

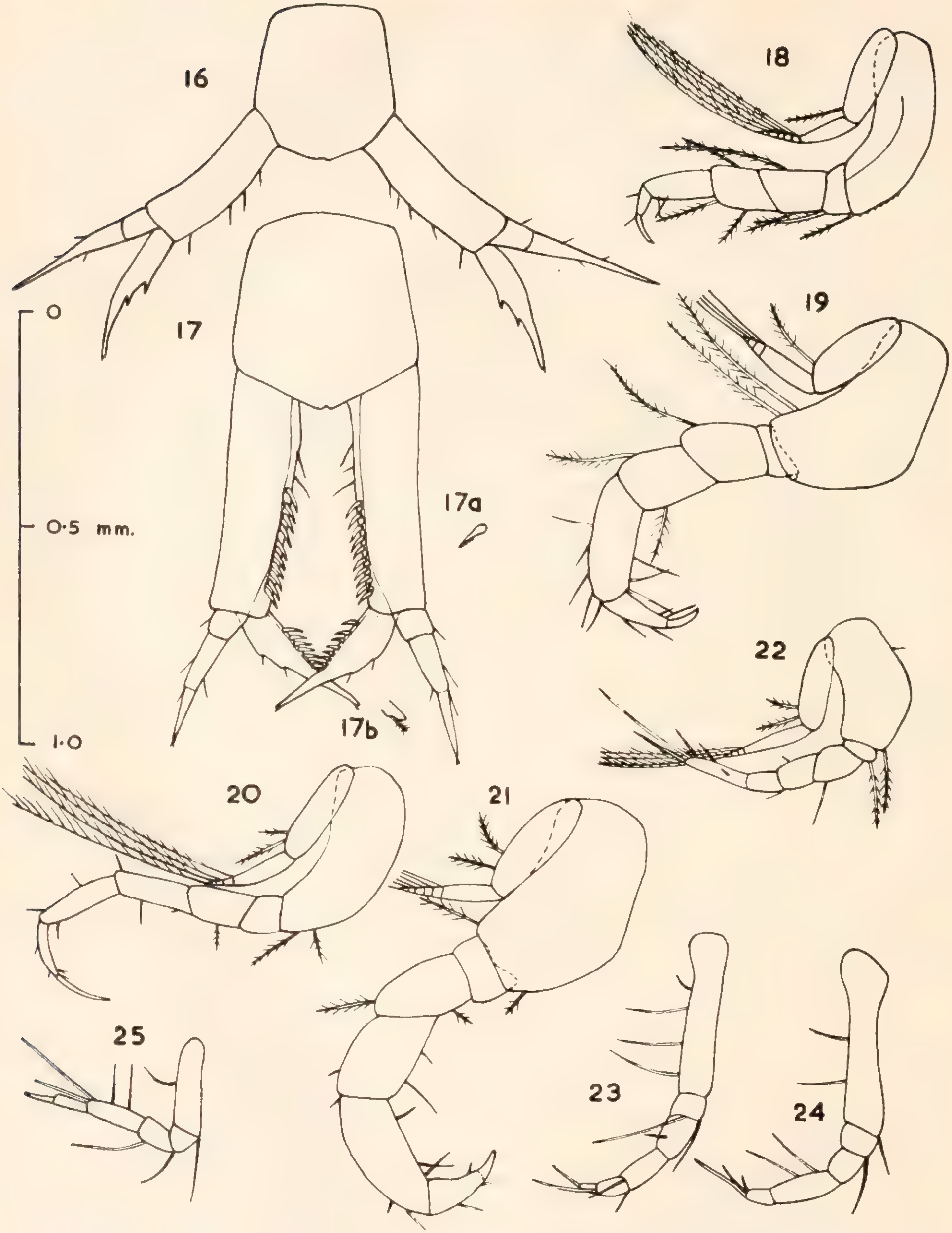

Figure 16. Female uropods.
Figure 17. Male uropods. Figure 17a. Same, spine of peduncle further enlarged.
Figure 17b. Same, tip of inner ramous further enlarged.
Figure 18. Female third maxilliped.
Figure 19. Male third maxilliped.
Figure 20. Female first peraeopod.
Figure 21. Male first peraeopod.
Figure 22. Female second peraeopod.
Figure 23. Female third peraeopod.
Figure 24. Female fourth peraeopod.
Figure 25. Female fifth peraeopod.

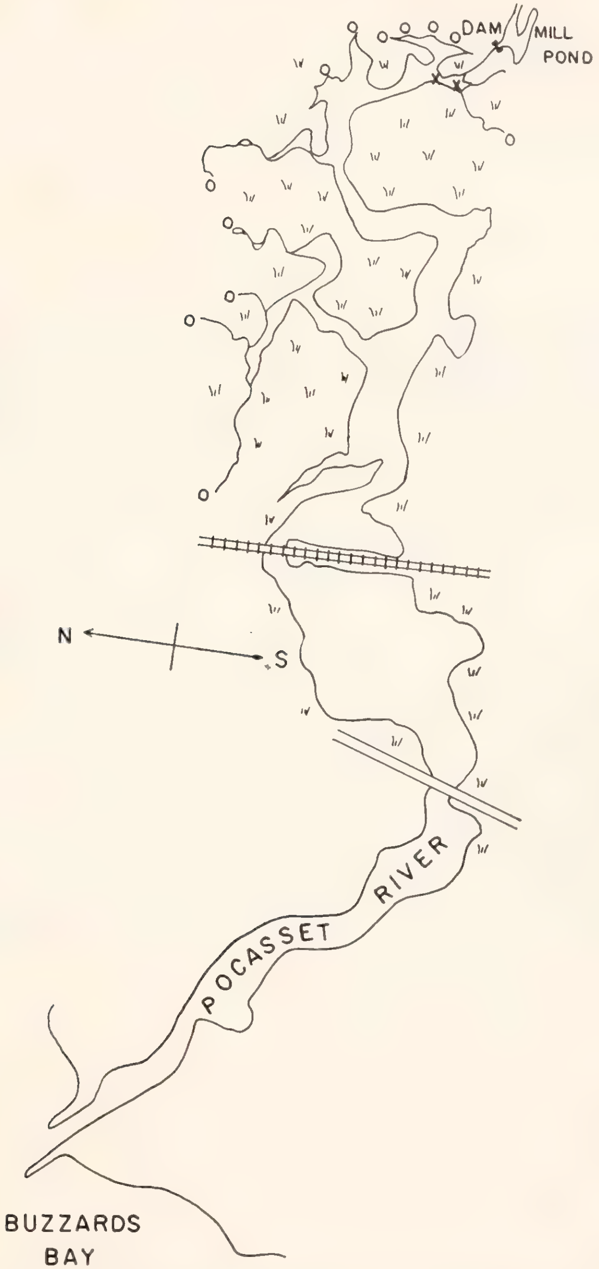
DAM
MILL POND
N
S
POCASSET RIVER
BUZZARDS BAY

FIGURE 26. Tracing of an airplane photograph of the Pocasset River, Cape Cod, Massachusetts taken on March 18, 1958 at 10:10 AM Eastern Standard time at an altitude of 2300 feet. The distance from the mouth of the river to the dam is approximately 1.4 miles. The X's in the headwaters indicate the type location of the cumaceans and the O's represent known locations of springs.

a pair was seen in a finger bowl containing algae which had been brought in to the laboratory from the field on August 18, 1958.

The composition of the substrate is unusual since it contains not only sand and gravel and plant débris but also a great many charcoal fragments and small pieces of iron slag. Supposedly the latter material was residue left from an iron foundry located on this site 73 years ago. Although porous, the substrate underlying the 1-cm. thick algal-detritus layer is quite hard and supports easily the weight of a man standing or walking on it.

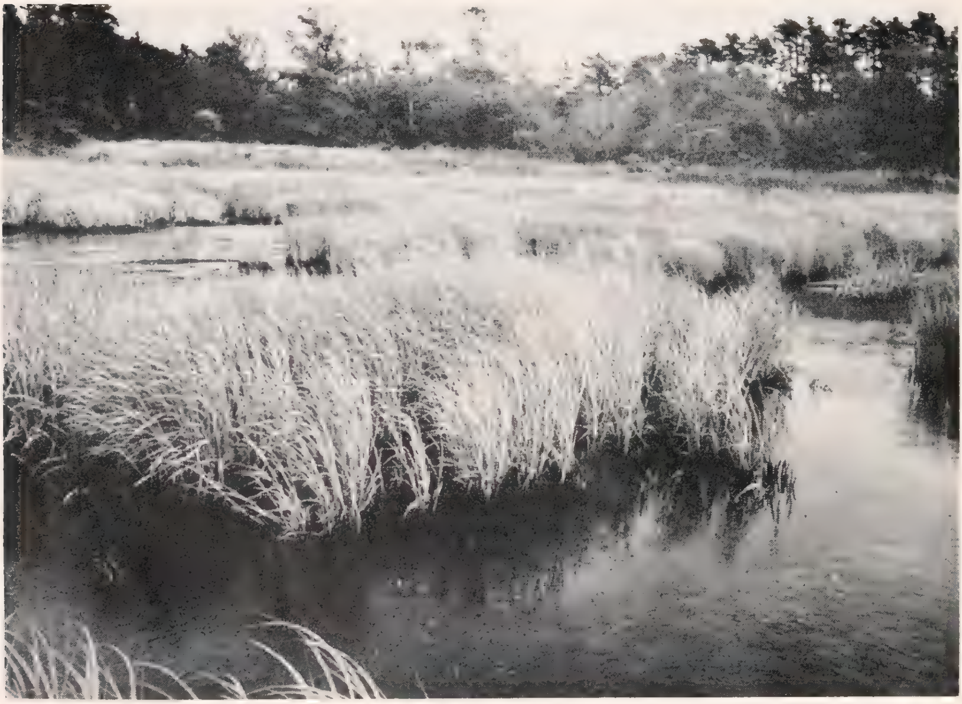

FIGURE 27. View of the Pocasset River looking upstream in an easterly direction at the type location. The cumaceans were found in the left foreground in an algal mat beneath shallow water and on the exposed flat above and to the left. These two areas are represented in Figure 26 by the more southern of the two X's. Photograph taken August 4, 1955 at low tide.

Although the small cumaceans were noted in collections from Pocasset River made from December 1957 to September 1958, there is no reason to believe that they are not present and active every month in the year.

General ecology. Associated with the cumacean and perhaps a source of food for it is the diatom, *Melosira* sp., which is "probably the dominant in terms of bulk and general distribution" (A. J. Bernatowicz, private communication). Also present are the blue-green alga, *Anabaena* sp., and, on pebbles, *Ulothrix* sp. Small numbers of the larger algae, *Monostroma* sp., *Ulva* sp., and *Enteromorpha* sp., are present while *Vaucheria* sp. lives on the mud among the adjacent *Spartina alterniflora* Loisel.

Living with the cumacean are two tanaids. The very common one is *Leptochelia dubia* (Kroyer) which has a breeding cycle similar to that of the cumacean, and less frequent is *Leptochelia rapax* (Harger). Two gammarids are also present in large numbers during the warmer months and these are *Gammarus tigrinus* Sexton and *Leptocheirus* sp. *Corophium lacustre* Vanhöffen is also in association with the cumaceans but it, unlike the gammarids, is quite patchy both as to distribution and numbers.

Just under the animal-algal association and sometimes entering it are the isopods, *Cyathura* sp., *Edotea* sp., and *Chiridotea almyra* Bowman. Of these only *Cyathura* sp. was ever found in appreciable numbers; however, since it is commonly found in densities of 1000–1400 per m.^{2}, it might be considered to be the dominant form in the upper reaches of Pocasset River where the cumaceans live. Often the ampharetid worm, *Hypaniola grayi* Pettibone, was found with the crustaceans as well as the spionid worm, *Scolecolepides viridis* (Verrill), which is present in largest numbers during the warmer months.

The only vertebrates regularly found with the cumacean were elvers of the American eel, *Anguilla rostrata* (Le Sueur). Examination of stomachs of small eels 8–10 cm. in length revealed that they ate cumaceans. Other fish in the same locality which eat small crustaceans and might well feed on cumaceans were the killifish, *Fundulus heteroclitus* (L.), the four-spined stickleback, *Apeltes quadracus* (Mitchell), and some small clupeids and other members of the herring family. The black duck and least sandpiper also feed in the area where the cumaceans live.

In all months of the year except March the cumaceans are dispersed, with only a few occurring in four-cubic inch cores of the algal-detritus layer. In March, however, as many as 50 were found in a sample of that size. Apparently the large increase is due to aggregation rather than to a sudden seasonal increase in total numbers.

In 1955 Bowman described the type habitat for the estuarine isopod, *Chiridotea almyra*, and he also listed the invertebrates living in association with it. The type locality was the Edisto River, S. C., with collections having similar habitats and associations from the Ogeechee River, Ga., and Haverstraw, N. Y. Pocasset River, because it possesses a very similar type of habitat and fauna, may represent a northern extension of the same type of tidal-marsh community.

It is well known that a number of species of Cumacea, all placed in the Pseudocumidae, occur in brackish or almost fresh water in the Caspian and neighboring regions, and species of *Cumella* have been found with other forms in the Black Sea in water of salinity about 18–21 ‰, but *Almyracuma proximoculi* is the first member of the Nannastacidae to be found in water of such low salinity as exists in its habitat at low tide. A few other species of Cumacea have been found in brackish water on the eastern coast of North America, especially in Chesapeake Bay, including *Mancocuma altera* Zimmer, *M. stellifera* Zimmer, *Cyclaspis pustulata* Zimmer, *C. varians* Calman, *Leucon americanus* Zimmer and *Oxyurostylis smithi* Calman (Zimmer, 1941), but these are all placed in other families.

Acknowledgments. For photographs of the Pocasset River: Airplane view from which tracing was taken, Mr. Carlyle Hayes of the Woods Hole Oceanographic Institution, and the type location, Dr. Charles Ray, Jr., Dept. of Biology, Emory University. For the identification of Crustacea, Dr. Thomas E. Bowman of the

Smithsonian Institution, U. S. National Museum, Dr. Milton A. Miller, Dept. of Zoology, University of California, Dr. Henry Werntz, The Biological Laboratories, Harvard University. For identification of the polychaete worms, Dr. Marian Pettibone, Dept. of Zoology, University of New Hampshire. For the identification of algae, Dr. A. J. Bernatowicz, Dept. of Botany, University of Hawaii.

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PURINES AND PTERIDINES FROM THE REFLECTING PIGMENT OF THE ARTHROPOD RETINA[1]

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Three sets of pigments are generally found in the crustacean retina, where they may undergo photomechanical movements under control of neurosecretory hormones (Kleinholz, 1936; Welsh, 1939; Brown *et al.*, 1952, 1953). The distal retinal pigment and the so-called proximal pigment of the retinular cells are dark pigments, presumed to be melanins or ommochromes, although few studies have been made of their chemical nature. The retinal reflecting pigment has been called guanine, but this identification has been based more on the analogy with the tapetal pigment occurring in the eyes of some vertebrates than on chemical study (Welsh, 1932; Kleinholz, 1936). I attempted, a score of years ago, to examine the chemical nature of this reflecting pigment of the crustacean retina, but, beyond gathering some information on solubility properties, efforts toward more specific characterization proved abortive because of limited amounts of available material.

Development within the past decade of techniques for isolation and examination of small amounts of biological material prompted a renewed attempt to identify this reflecting material. This initial study, part of which has been reported in preliminary form (Kleinholz, 1955), was done on the lobster, *Homarus americanus*, and the chelicerate, *Limulus polyphemus*.

METHODS

Eyestalks of *Homarus* were usually removed before the rest of the animal was turned to other purposes. The eyes of *Limulus*, together with adjacent tissue, were excised from animals immobilized by bleeding. Immediately after removal the eyes were placed in 95% ethanol for 2 to 4 days for hardening, after which the retinas of *Homarus* were cut from the stalks while, in *Limulus*, the extraneous tissue was dissected away from the eye. The ethanol was changed frequently until no more color was leached from the retinas.

Retinal reflecting pigment in *Homarus* does not undergo photomechanical changes and occurs as a compact layer distal to the fenestrated basement membrane, as well as in substantial deposits proximal to this membrane (Fig. 1). Initially, the reflecting layer was exposed by removing and discarding these proximal deposits and adjacent tissue; material from the reflecting layer was then scraped free in ethanol and concentrated by centrifugation. After it was found that the chromatographic results were qualitatively the same, these deposits of reflecting pigment

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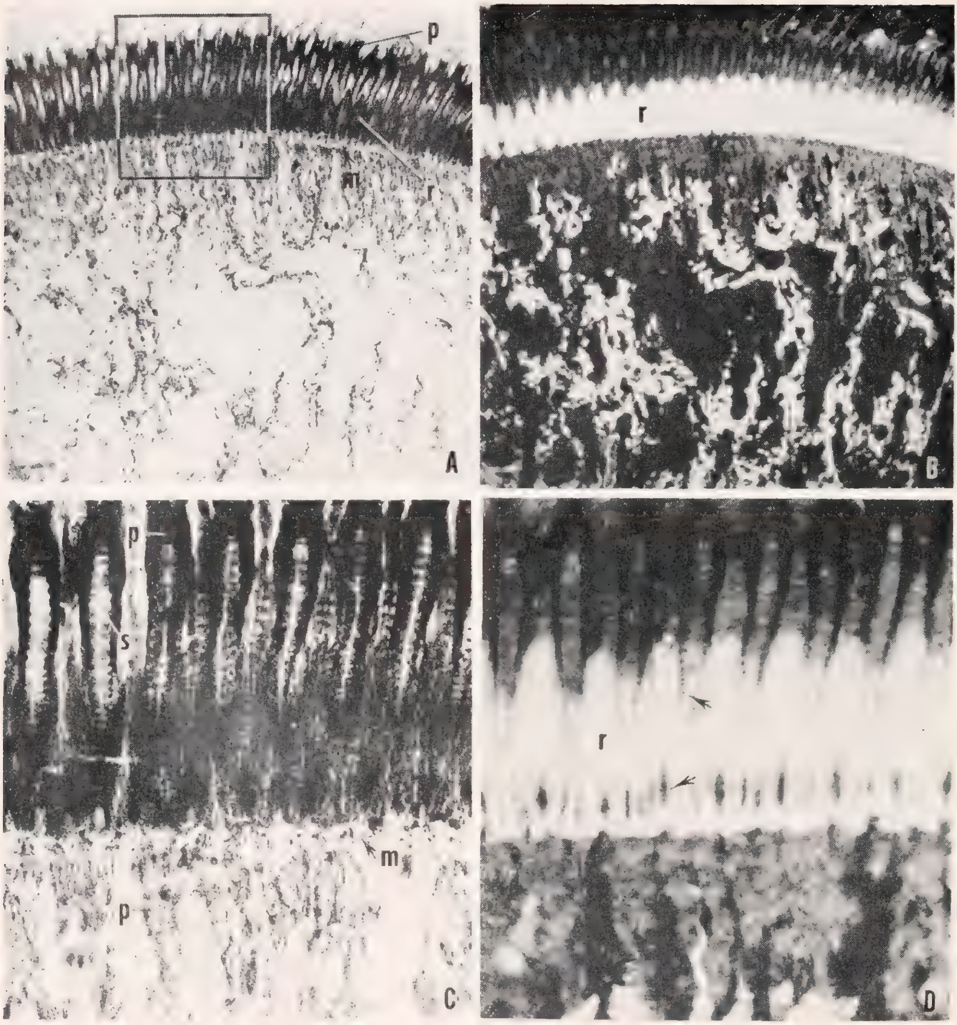

FIGURE 1. All the photographs are of a longitudinal section through the eye of *Homarus* and show the proximal portion of the retina. The bottom of each figure is proximal to the body; the top of the figure is distal from the body.

FIGURE 1A. Bright-field illumination; the proximal pigment and the layer of reflecting pigment above the fenestrated basement membrane surround the rhabdomes. Granules of both proximal pigment and reflecting pigment also occur below the basement membrane, but these are not readily distinguishable from each other.

FIGURE 1B. Dark-field illumination of the same region seen in Figure 1A. The layer of reflecting pigment distal to the fenestrated basement membrane, and the deposits of this pigment proximal to the basement membrane are now readily evident. Comparison of the distribution patterns of the pigments below the basement membrane in the two prints permits some differentiation between granules of reflecting pigment and of proximal pigment.

FIGURE 1C. The rectangular region marked in Figure 1A shown under higher magnification by bright-field illumination.

proximal to the basement membrane also were combined with the scrapings from the reflecting pigment layer.

In the case of *Limulus*, reflecting pigment is located distally in the eye. The intervening retinal melanin was exposed, chipped away with a small scalpel, and discarded. In a few instances most of this retinal melanin was dissolved by immersing the eye for an hour in ethylene chlorohydrin; the treated retinas were then washed in a few changes of ethanol. Either of these methods of removing the melanin exposed the reflecting pigment which was then scraped free and concentrated by centrifugation. Masses of white material, similar in appearance to the reflecting pigment, and described by some authors as "rudimentary eyes," are closely associated anatomically with the lateral and median eyes of *Limulus;* these, too, were removed for study.

The reflecting pigments and associated tissue were ground and extracted from 1 to 6 hours in a micro-centrifuge tube with 0.1 ml. per retina of one of the following alkaline solutions: 1% NaOH; 1% LiOH; 0.5 N NH_4OH; 0.06% Li_2CO_3; 0.2 *M* borate buffer at pH 9.2; or a solution of 50% ethanol containing 2% NH_4OH. The tubes were centrifuged and samples of the supernatant solution as well as samples of standard purine solutions were applied with a micro-pipette to sheets or strips of Whatman No. 1 filter paper for subsequent chromatography or electrophoresis.

Either ascending or descending development was used with a wide variety of solvent mixtures, such as are listed by Block, Durrum and Zweig (1955) and by Viscontini, Schmid and Hadorn (1955). The most useful solvent systems were: (1) water-saturated n-butanol:formic acid = 9:1; (2) pyridine:ethyl acetate:water = 4:3:3; (3) isoamyl alcohol saturated with 5% disodium hydrogen phosphate (with a layer of each in the chromatography chamber); (4) water-saturated collidine; (5) 3% aqueous ammonium chloride; (6) n-butanol:acetic acid:water = 8:2:2, followed by a second development in the same direction with acetone:n-butanol:water = 8:1:1. After development the paper was dried and examined in short-wave ultraviolet light (Mineralight Model V-41 lamp, manufactured by Ultraviolet Prod. Inc.) and the spots outlined with pencil. Tentative identifications of the components of the reflecting pigment were made by comparing the distances the component spots migrated with the distances migrated by the spots of reference standards. Spots developed from reflecting pigment were cut out and eluted in 0.1 N NaOH or 0.1 N HCl. The identification was then verified by determining the ultraviolet absorption spectra of these eluates in a Beckman spectrophotometer and comparing them with spectra of the known standards. In a large number of cases developed chromatograms were also treated according to the method of Vischer and Chargaff (1948) whereby purine spots are made visible as a black mercuric sulfide complex. The latter procedure revealed overlapping or masking of components when they occurred, and thus indicated need for development in different solvent systems.

Paper electrophoresis was used primarily in resolving one of the pteridines

FIGURE 1D. Dark-field illumination of the region shown in Figure 1C. Arrows point to granules of dark proximal pigment intermingled with the reflecting pigment layer. Strands of reflecting pigment at the bottom of the print aid in recognizing this pigment in Figure 1C. *m*, fenestrated basement membrane; *p*, proximal pigment; *r*, reflecting pigment; *s*, rhabdome.

which could not be satisfactorily separated from the other components of reflecting pigment by paper chromatography. Samples, about 0.1 ml. in volume, of ethanol-ammonia extract of lobster retina were applied to paper strips which were then developed at 375 volts or 500 volts for 18 to 20 hours in the LKB or the Spinco instrument. The buffer was 0.04 *M* boric acid and 0.01 *M* borax at pH 8.6. After the strips were developed and dried, the blue-fluorescent segments, which had migrated toward the cathode, were cut out and eluted in 0.1 N HC1 or in 0.1 N NaOH for subsequent spectrophotometry.

Initial studies on solubility of the reflecting pigment of *Homarus* were made on histological sections cut at 10 microns from paraffin-embedded retinas. The mounted sections were de-waxed with xylene and re-hydrated before testing with the various solvents. The murexide and enzymatic tests were made on small amounts of reflecting pigment which had been removed as described. The methenamine-silver reaction of Gomori (1952) was used as a histochemical test for uric acid.

RESULTS

A. Nature of the reflecting pigment of Homarus

Reflecting pigment was dissolved from sections of lobster retina within 30 minutes after immersion in 1 N solutions of specific acid (hydrochloric, acetic, nitric or sulfuric) or of specific alkali (ammonium hydroxide, sodium hydroxide, 0.1% aqueous solutions of sodium carbonate or sodium bicarbonate). At 60° C. the reflecting pigment dissolves within an hour in glycerine or ethylene glycol or ethylene glycol monoethyl ether. When, however, these same solvents are used at room temperature, one finds little visible solution in glycerine, partial solution in ethylene glycol monoethyl ether, and complete solution in ethylene glycol. The reflecting pigment is partially dissolved from sections remaining overnight in 95% ethanol but showed no discernible solution in absolute ethanol. These solubilities differ in several important respects from those reported for guanine by Millot (1923). Thus, according to Millot, guanine is insoluble in ammonium hydroxide or acetic acid, whereas the reflecting pigment of *Homarus* is soluble in both these solutions. Gwilliam (1950) also reports solubilities of retinal reflecting pigment of the crab, *Hemigrapsus oregonensis*, that fail to agree with those of guanine.

The residue obtained by evaporating to dryness a dilute lithium carbonate extract of *Homarus* reflecting pigment gives positive murexide but negative or faint, dubiously-positive Weidel reactions. Guanine, uric acid, xanthine and its methyl derivatives give positive murexide reactions (Lison, 1936). Millot (1923) reports that guanine and xanthine, but not uric acid, react positively to the Weidel test; adenine and hypoxanthine, among the other common purines, are reported to give neither murexide nor Weidel reactions. Comparison of these reported results with the findings for *Homarus* casts doubt on the reflecting pigment's being guanine and indicates, instead, that the reflecting pigment of the lobster may be uric acid.

A histochemical test depending on an argentaffin reaction between uric acid and methenamine-silver (Gomori, 1952) proved positive for the reflecting pigment of *Homarus*. Argentaffin reactions, particularly in neutral solution, have been criticized (Lison, 1936) because positive reactions are also given by calcium carbonate and phosphate, if present. In this study, however, exposure of sections to methenamine during incubation is supposed to bring about ready solution of such calcifications.

More specific identification of uric acid in the reflecting pigment was made by paper chromatographic resolution of mixtures after incubation with uricase (Nutritional Biochemicals Corp.). Preliminary exploration showed that 1 to 5 μgm. of uric acid in 5 μl. of 0.5% lithium carbonate solution are detectable when the n-butanol-formic acid solvent system and the Vischer-Chargaff (1948) visualization method are used. Uric acid and 5 μl. of a solution containing the reflecting pigment of one lobster retina in 0.1 ml. showed similar R_f indices (0.14 to 0.17) with this same solvent system.

The reflecting pigment of 20 lobster eyes, dissolved in 0.5 ml. of dilute lithium carbonate solution, was mixed with 50 mg. of uricase, 0.5 ml. of 0.05 *M* borate buffer at pH 9.2, and 0.5 ml. of toluene. A 5-μl. sample of this mixture was removed for application to paper within 5 minutes (zero time). This mixture was gassed with oxygen and incubated at 38° C. Thereafter, at intervals of 0.5, 1, 2, 4, and 6 hours, 5-μl. aliquots were removed and applied to paper; a 5-μgm. sample of uric acid to serve as a reference standard was applied to the same sheet of paper which was then developed in butanol-formic acid solvent. Treatment of the developed chromatogram by the Vischer-Chargaff method revealed the purine as black spots with an R_f index of 0.15 for the reference standard and also for those aliquots taken at 0-, 0.5- and 1-hour intervals. The intensity of the spots decreased with time of incubation with uricase. The sample taken after 2 hours of incubation showed an R_f index of 0.14 and was very faint. No spots were present for the 4-hour and 6-hour samples. Because of the specificity of uricase in the oxidation of uric acid, these results may be considered a satisfactory demonstration of the presence of uric acid in the retinal reflecting pigment of *Homarus*. Examination of these chromatograms revealed an additional faint spot distal to each of the corresponding retinal uric acid spots; this faint spot was not present above the uric acid standard. The possible presence of other purines besides uric acid was indicated by this observation.

This possibility was explored by first examining chromatograms developed in butanol-formic acid solvent in ultraviolet light, and then using the mercuric nitrate-ammonium sulfide visualization method for purines. When this was done, the results diagrammed in Figure 2 were obtained for the lobster. The diagram shows the presence of three apparent purines, one of which is uric acid, and two fluorescent compounds. For subsequent reference, these spots are labelled, starting from the baseline on the chromatogram, as Fluorescent 1, Absorbent 1 (uric acid), Fluorescent 2, Absorbent 2, and Absorbent 3.

B. Further identification of the retinal compounds

The two fluorescent compounds of the reflecting pigment were believed to be pteridines which have been reported present in the eyes of vertebrates (Pirie and Simpson, 1946; Hama, 1953) and of crustaceans (Busnel and Drilhon, 1948). After chromatographic development of retinal pigment samples and aliquots of known purines and xanthopterin as reference standards in a variety of solvent systems, the R_f indices of the components were compared. In this way, four of the five spots of Figure 2 were identified: Absorbent 1 is uric acid; Fluorescent 2 is xanthopterin; Absorbent 2 is xanthine; and Absorbent 3 is hypoxanthine. The linear sequence of the spots, starting from the baseline on the chromatogram, may vary strikingly with different solvent systems (Fig. 3). Advantage was taken of this property to make the final verification of the above-mentioned identifications. Well-resolved spots, not masked or overlapped by other components, were cut out, eluted in 0.1 N HCl, and the absorption spectrum of the eluate determined. The maxima of the spectra obtained for spots identified as uric acid, xanthine, and hypoxanthine corresponded with those reported by Dorough and Seaton (1954).

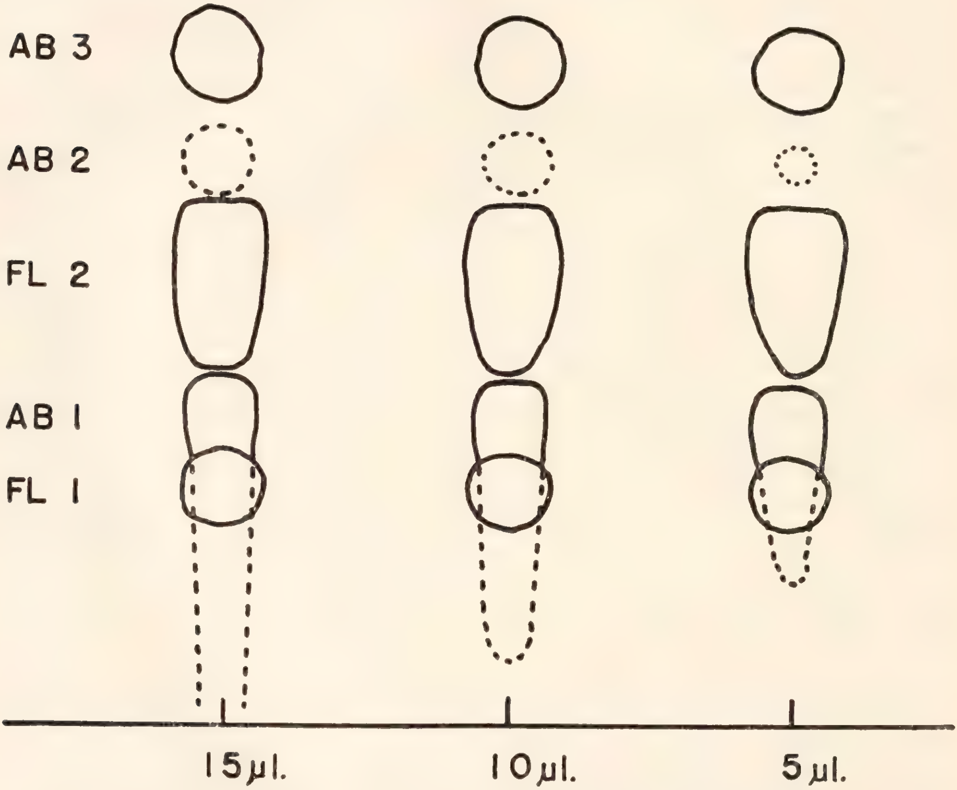

FIGURE 2. Tracing of a chromatogram of retinal reflecting pigment of *Homarus*, developed in butanol-formic acid solvent. The resolved spots were first outlined in ultraviolet light and then were treated to make the purine spots visible; the broken lines indicate boundaries made evident after this latter treatment. The size of the sample applied to the paper is given in microliters. The labels to the left identify and describe the appearance of the spots in ultraviolet light: FL, fluorescent; AB, absorbent. See text.

The absorption spectrum of the retinal component identified by R_f index as xanthopterin was determined after similar elution from a chromatogram developed in butanol-formic acid solvent. This is compared with the spectrum obtained from eluates of xanthopterin used as a reference standard on a paper chromatogram

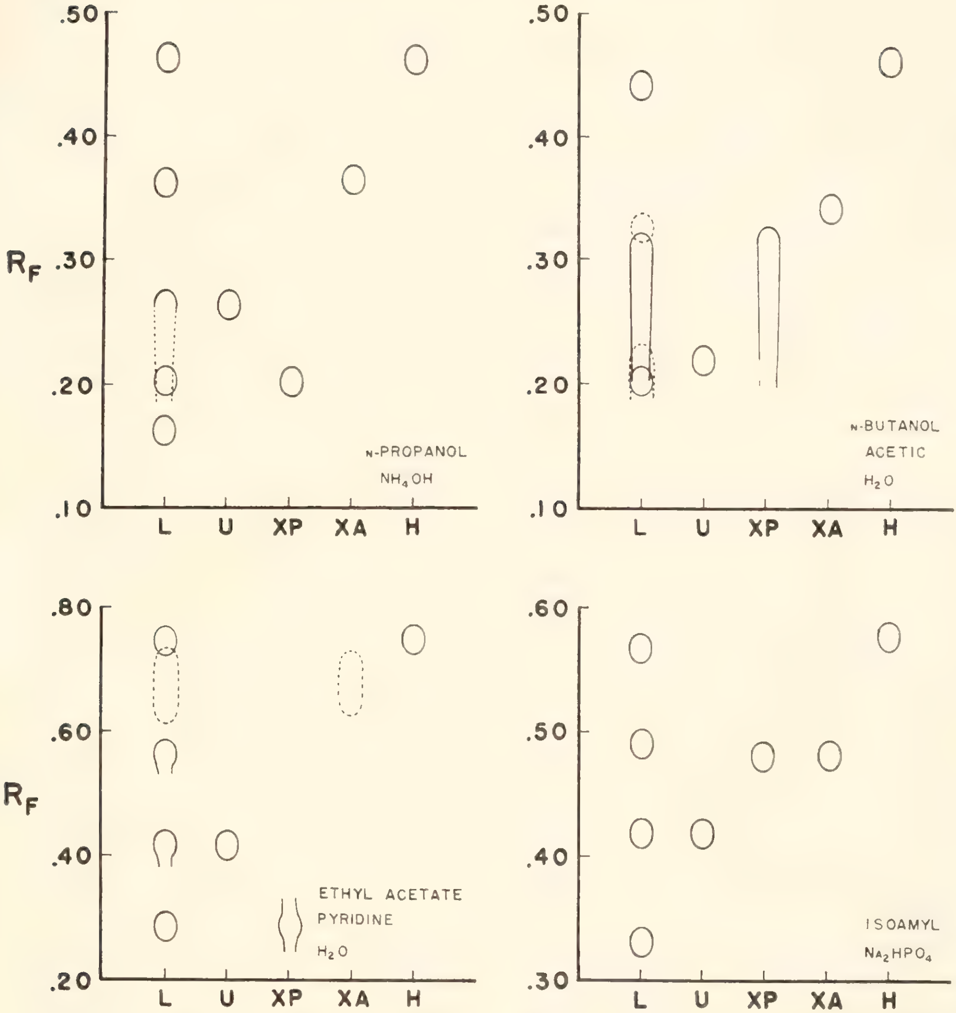

FIGURE 3. Diagrams showing the tentative identification of four of the five components of the reflecting pigment of *Homarus* arrived at by comparing the R_f indices of the components with those of reference standards. The solvent system is indicated on each diagram. Broken lines represent the boundaries of components not evident on examination in ultraviolet light but which became visible after formation of the mercuric sulfide complex. L, reflecting pigment of *Homarus;* U, uric acid; XP, xanthopterin; XA, xanthine; H, hypoxanthine.

(Fig. 4). The maxima of the reference xanthopterin at 230 mμ, 259 mμ, and 355 mμ agree with the maxima reported for xanthopterin by Elion and Hitchings (1947). The spectrum of xanthopterin from the retina shows similar maxima at 231 mμ, 261 mμ, and 355 mμ, although the geometry of the retinal spectrum differs somewhat from that of the reference standard. The basis of this difference is not understood.

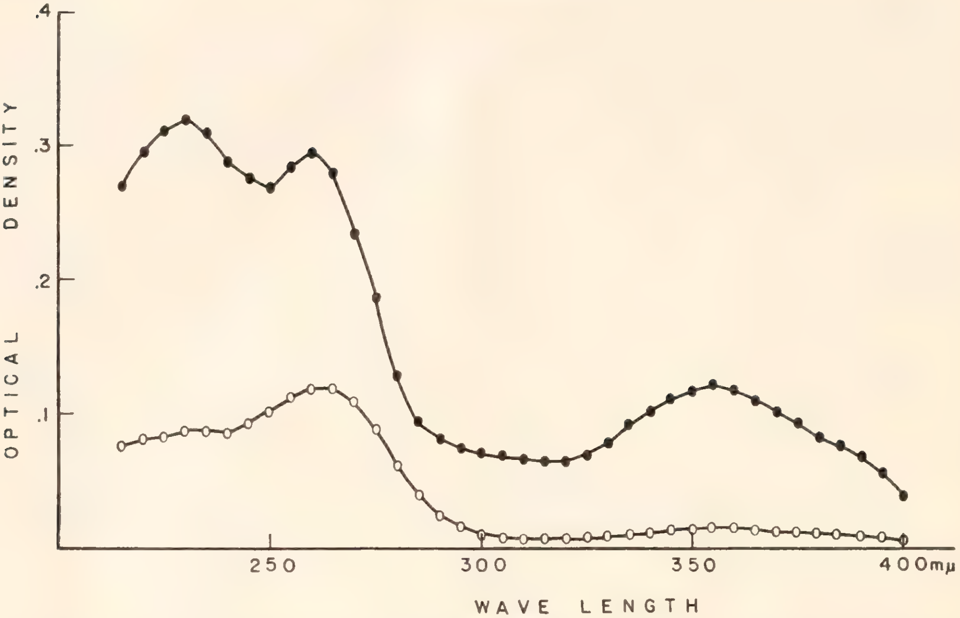

FIGURE 4. Absorption spectra of reference xanthopterin (upper curve) eluted from paper chromatogram and of Fluorescent 2 component (lower curve) from reflecting pigment of *Homarus*.

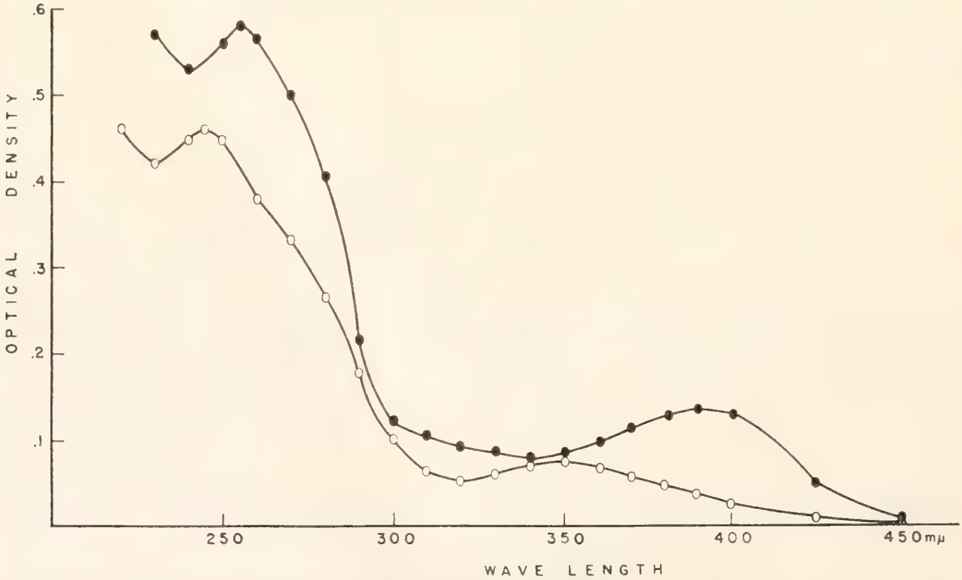

FIGURE 5. Absorption spectra of unidentified Fluorescent 1 component of lobster reflecting pigment. Upper curve is the eluate from the paper in 0.1 N NaOH; lower curve is the eluate in 0.1 N HCl.

C. *The unidentified fluorescent component*

There remains to be considered the unidentified component of *Homarus* reflecting pigment labelled Fluorescent 1 in Figure 2. The rarity of many pure pteridines limited the number available for use as chromatographic standards; the R_f indices of the few pteridines used for this purpose failed to give a satisfactory match with the index for Fluorescent 1 in a variety of solvent systems. Attempts to isolate this component in sufficient concentration for subsequent spectrophotometry, as was done with the other retinal components, were generally frustrated by contamination due to streaking or tailing of the other constituents.

Fluorescent 1 was finally isolated by paper electrophoresis, and was eluted in 0.1 N HCl or in 0.1 N NaOH, as described in Methods. The absorption spectrum of the eluate in acid showed maxima at 245 mμ and 353 mμ; in alkali, these maxima were shifted to 255 mμ and 390 mμ (Fig. 5).

D. *Specific localization within the retina*

It cannot be stated with complete certainty in which part of the lobster retina the five purines and pteridines are specifically localized. The evidence described above indicates that uric acid is most probably a component of the reflecting layer of retinal pigment, as may also be the two other purines, xanthine and hypoxanthine. The two pteridines may be more widely distributed among the retinal components. Busnel and Drilhon (1948) found several substances, detectable by fluorescence microscopy, in the crustacean retina. These fluorescent materials not only are closely associated with the proximal pigment but also occur in the regions of the reflecting and distal pigments.

It is apparent from Figure 1 (C and D) that, although most of the proximal pigment in light-adapted retinas has migrated distal to the reflecting pigment layer, proximal pigment granules still remain intermingled with and below this layer. The preparation of reflecting pigment for chromatography unavoidably included some of these proximal pigment granules. However, chromatography of preparations of reflecting pigment, previously washed with ethylene chlorohydrin to remove the traces of dark proximal pigment, showed the presence of the two pteridines obtained with untreated reflecting pigment. Thus, while the above observations are presumptive evidence for localization of the pteridines in the reflecting pigment, the possibility of their occurring also in the other retinal pigments cannot be excluded.

E. *Retinal reflecting pigment in Limulus*

Reflecting pigment from the lateral and median eyes of *Limulus* was obtained as described under Methods. The deposits of white material of so-called rudimentary eyes, located in the postero-medial region of each lateral eye, as well as similar material associated with the median eyes, were dissected free. Each of these was dissolved separately in 0.5% NaOH. Samples of the solutions were applied to paper and developed, along with a series of purine reference standards. The solvent systems were butanol-formic acid; water-saturated collidine; and butanol-water-morpholine-diethylene glycol. Examination of the chromatogram in ultraviolet light generally revealed a single quenching spot whose R_f index was the same as that of guanine. A faintly bluish-fluorescing spot was also evident in one case but was not observable in any of the other chromatograms. Chromatograms treated by the Vischer-Chargaff method confirm the coincidence of R_f indices for the reference guanine and reflecting pigment from lateral, median, and rudimentary eyes.

The spots quenching ultraviolet light, obtained with reflecting pigment from a lateral eye, were cut from a chromatogram developed in butanol-formic acid and were eluted overnight in 1% NaOH. The spectrum of this eluate had a maximum at 275 mμ, in agreement with that reported by Hotchkiss (1948) for guanine.

I am indebted to Profs. C. M. Williams and J. H. Welsh for helpful suggestions and critical comments on the manuscript.

SUMMARY

1. The chemical nature of the retinal reflecting pigment was studied in *Homarus* and in *Limulus.* In crustaceans the reflecting pigment has been thought to be guanine, but the solubility and chemical properties of this pigment from *Homarus* do not agree with those for guanine.

2. Use of paper chromatographic methods shows the presence of five substances in the reflecting pigment of *Homarus,* three of which are absorbent or quenching in ultraviolet light and two of which are fluorescent.

3. Histochemical treatment with methenamine-silver and incubation studies with uricase identify one of the three ultraviolet-absorbent compounds as uric acid. Comparisons of R_f indices of the other two ultraviolet-absorbent compounds with those of reference purines show them to be xanthine and hypoxanthine. Identifications of all three were verified by determining the ultraviolet absorption spectra of the retinal purines eluted from paper chromatograms.

4. One of the two fluorescent components of *Homarus* reflecting pigments is xanthopterin, identified both by its R_f indices after chromatographic development in a variety of solvent systems, and by its absorption spectrum. The second fluorescent compound, probably a pteridine, has not been identified, but its absorption spectrum shows maxima at 245 mμ and 353 mμ in 0.1 N HCl; in alkali these maxima are shifted to 255 mμ and 390 mμ.

5. Retinal reflecting pigment from *Limulus* is guanine.

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THE RESPIRATORY ENZYMES OF DIAPAUSING SILKWORM PUPAE: A NEW INTERPRETATION OF CARBON MONOXIDE-INSENSITIVE RESPIRATION[1]

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The respiration of most organisms is inhibited in large measure by carbon monoxide. This indicates that cytochrome oxidase is the main terminal enzyme in electron transfer (Warburg, 1949; Keilin and Slater, 1953). But as is well known to students of insect physiology, the respiration of many diapausing insects is remarkably insensitive to cyanide, carbon monoxide, and other inhibitors of cytochrome oxidase. The significance of this insensitivity was recently discussed by Harvey and Williams (1958a, 1958b) as a result of studies on the heart of diapausing pupae of the Cecropia and Polyphemus silkworms. Quite independently we have carried out a detailed study of another aspect of this phenomenon (Kurland, 1957). Our attention has centered, not on a single organ such as the heart, but on the respiration of the whole insect. These two investigations prove complementary in the analysis of the problem as a whole.

Carbon monoxide-insensitive respiration in insects was first detected by Bodine and Boell (1934a, 1934b) who reported that the oxygen consumption of diapausing eggs of the grasshopper *Melanoplus* was not inhibited by carbon monoxide. Later, Allen (1940) showed that the cytochrome *c* oxidase activity of these diapausing eggs was high, despite the insensitivity of their respiration to both carbon monoxide and cyanide. He concluded (p. 162) that the "rates of oxygen consumption of pre-diapause, diapause, and very early post-diapause eggs are independent of the relative amounts of cytochrome oxidase." An important clue to the reconciliation of the CO-insensitivity of diapausing *Melanoplus* eggs with the simultaneous presence of cytochrome oxidase was provided by Bodine and Boell (1936, 1938). They discovered that 2,4-dinitrophenol (DNP) increased the respiration of diapausing eggs and that this increased respiration was inhibited by carbon monoxide and cyanide. Unfortunately, the significance of this observation could not be fully comprehended because the mechanism of DNP action was not explained until a decade later (Loomis and Lipmann, 1948).

As the result of an intensive investigation of the CO-insensitivity of pupal respiration in the giant silkworm *Hyalophora cecropia,* Schneiderman and Williams (1952, 1954a, 1954b) concluded that the cytochrome *c* oxidase system was not functioning in most tissues of the diapausing pupa, although it functioned at all other stages in the life history. Their arguments have recently been summarized (Lees, 1956; Schneiderman, 1957). They suggested that pupal respiration was mediated by an autoxidizable flavoprotein or a heme-containing enzyme insensitive to carbon monoxide. This explanation was supported by the observations of Shappirio and Williams (1953), Shappirio (1954), and Pappenheimer and Williams (1953, 1954) who reported the existence of a new autoxidizable cytochrome component (*e* or b_5) in Cecropia pupae. Also, Chefurka and Williams (1952) reported an increased amount of flavoprotein in pupal tissues. However, there was no evidence to indicate that the new cytochrome or the flavoprotein functioned as a terminal oxidase in the pupal respiratory chain.

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[1] This study was aided by Grant H-1887 from the National Heart Institute, U. S. Public Health Service. Several of the experiments were drawn from a thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Arts with Honors in Zoology from Cornell University.

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The experiments reported here continue these earlier studies and were prompted, in part, by recent advances in our understanding of electron transfer in the cytochrome system (*cf.* review by Chance and Williams, 1956). We examined the effects on respiration of two inhibitors of cytochrome oxidase, carbon monoxide and sodium azide, and also of antimycin A, a potent inhibitor of the DPNH-cytochrome *c* reductase system. In addition we studied the effects of 2,4-dinitrophenol which dissociates phosphorylation from oxidation. It was hoped that a study of the action of these rather specific inhibitors on pupae in various metabolic states might permit a decisive definition of the terminal oxidase of diapausing pupae. This objective has been achieved. The results of the present study, coupled with the recent findings of Harvey and Williams (1958b), have enabled us to identify this oxidase as cytochrome oxidase and thus contradict earlier conclusions. The experiments also reveal some new biochemical peculiarities of the diapause condition.

MATERIALS AND METHODS

1. *Experimental animals*

Diapausing pupae of *Hyalophora (Platysamia) cecropia* (4 to 6 gm.), *Callosamia promethea* (1½ to 2½ gm.), *Samia cynthia* (1½ to 3½ gm.) and *Antheraea (Telea) polyphemus* (4 to 6 gm.) were used as experimental animals. In our experience diapausing pupae of these four species of closely related saturniid moths behave in virtually identical fashion in respiration experiments and hence we have used them interchangeably. The animals were reared under field conditions or collected in nature and were stored at 25° C. for a minimum of four months before use in experiments. One group of Cynthia and Promethea pupae was maintained at 5° C. for several months and then returned to 25° C., whereupon their brains were removed. This brain removal put the pupae in a state of permanent diapause (Williams, 1946) and, after three months at 25° C., these animals behaved in experiments like normal unchilled diapausing pupae. Only pupae displaying a relatively constant respiratory rate over a period of at least six hours were used in experiments. Also, since it was shown by Schneiderman and Williams (1954a) that cellular respiration of the pupal abdominal muscles is mediated by cytochrome oxidase, pupae showing excessive muscular activity were excluded.

2. *Measurement of respiration*

The present investigation is based on more than 2000 respiratory measurements performed on about 500 pupae. Rates of oxygen consumption were determined manometrically according to techniques described previously (Schneiderman and Williams, 1953a). Measurements were carried out in 50-cc. vessels equipped with venting plugs and adapters for use with standard Warburg manometers.

3. *Gas mixtures*

In some experiments, pupae were exposed to various gas mixtures while enclosed in the Warburg vessels. Commercial gases were purified and gas mixtures prepared and analyzed by methods described previously (Scheiderman and Williams, 1954a). All of the experiments were performed at atmospheric pressure. The vessels were periodically re-flushed during the course of the experiments, a maneuver which prevented any significant reduction of oxygen tension within the vessels. Appropriate control vessels were run in all experiments to take into account the manometric effect of reactions between carbon monoxide and the alkali.

4. *Reagents*

Sodium azide and 2,4-dinitrophenol were reagent grade. Crystalline antimycin A, obtained from the Wisconsin Alumni Research Foundation, was dissolved in aqueous ethanol. The final dilutions of antimycin A injected into the pupae were uniformly in 1% ethanol solutions.

Previous to injection, the pupae were anesthetized with carbon dioxide. In our experience the respiration of diapausing pupae is not significantly affected by thirty minutes of carbon dioxide anesthesia. Approximately 0.1 cc. of solution was injected *via* a 26-gauge needle into each pupa. The final concentrations within the animal were calculated on the basis of a pupal water content of 70 per cent. Respiration was measured for a minimum of three hours after injection.

5. *Interpretation of inhibitor experiments*

The act of piercing merely the skin of a diapausing pupa with a fine hypodermic needle causes a prompt stimulation of respiration for several hours. This is followed by a subsequent slow rise in respiration—injury respiration (Schneiderman and Williams, 1953a, 1953b). Hence in interpreting inhibitor experiments, it is necessary to separate the effects of injury from those of the chemical injected (Scheiderman and Williams, 1954a). This can best be accomplished by comparing experimental pupae with control pupae injected with a corresponding volume of the solvent used, *e.g.,* 1% ethyl alcohol, distilled water, etc. Furthermore, it is simplest to make comparisons soon after injection, before injury respiration increases to high levels and possibly before the injected chemical is detoxified or otherwise metabolized. In most of the inhibitor experiments to be reported, the pupae had a very low basal metabolic rate and simple injection commonly doubled their oxygen consumption.

6. *Injury*

Pupae were anesthetized with carbon dioxide. Injuries were made either by removing a rectangle of pupal cuticle and underlying hypodermis from the face or by excising the pupal legs. The wounds were then covered with plastic windows sealed in place with paraffin. A few crystals of streptomycin sulfate and phenylthiourea (a 1:1 mixture) were placed in the wounds to prevent infection and to prevent darkening of the blood by tyrosinase (Williams, 1952; Schneiderman and Williams, 1953a).

EXPERIMENTAL RESULTS

1. *Diapause respiration*

A. *The development of CO-insensitive respiration after pupation*

The effects of carbon monoxide on the respiration of newly pupated Cecropia silkworms were observed at intervals over a ten-day period. The pupae were exposed first to a nitrogen-oxygen mixture and then to a carbon monoxide-oxygen mixture. The results, as well as details of the procedure, are recorded in Table I. As the pupae aged they exhibited a gradual decrease in their respiratory rate which was accompanied by a marked decrease in the fraction of respiration sensitive to carbon monoxide. Thus while immediately after pupation half their respiration was inhibited by carbon monoxide, 200 hours later less than 20 per cent was CO-sensitive.

TABLE I

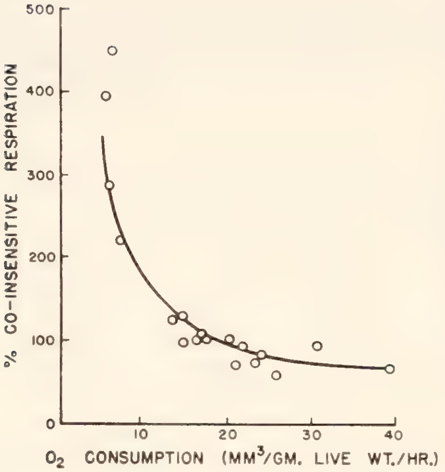
*The development of CO-insensitive respiration in four newly molted Cecropia pupae**

Age after pupation (hrs.)	Respiration in nitrogen mixture (mm.^{3}/gm./hr.)	% insensitive respiration
5	34	59
29	26	49
197	7	92
6	37	51
30	28	69
198	7	86
19	26	47
43	25	74
211	7	80
19	26	57
43	23	55
211	10	80

* Pupae were exposed for three hours to an atmosphere of 6 per cent oxygen and 94 per cent nitrogen, and then for three hours to an atmosphere of 6 per cent oxygen and 94 per cent carbon monoxide. To calculate per cent insensitive respiration, oxygen consumption in the carbon monoxide mixture was compared to oxygen consumption in the nitrogen mixture.

B. *The CO-insensitivity of respiration of diapausing pupae*

Figure 1 records the per cent of CO-insensitive respiration for a large number of pupae with different basal rates of oxygen consumption. The data show that as oxygen consumption increases, respiration becomes increasingly sensitive to carbon monoxide. However, it is of special interest that even when carbon monoxide inhibited the respiration of diapausing pupae it rarely inhibited more than 20 per cent of their total respiration, and for pupae whose basal respiration was between 15 and 20 mm.^{3}/gm. live wt./hr., the respiration appeared to be unaffected by carbon monoxide. It is also noteworthy that carbon monoxide appeared to stimulate the respiration or at least the *gas uptake* of pupae whose basal oxygen consumption was less than 15 mm.^{3}/gm. live wt./hr. We have duplicated these results in numerous experiments with Cynthia, Polyphemus and Promethea pupae. In all cases the apparent stimulation was greatest for pupae with low basal respiratory rates and possible explanations for this phenomenon will be offered in the Discussion. But for the present, suffice it to note that in the presence of carbon monoxide the *gas uptake* of pupae which have low respiratory rates in air is markedly increased and that this fact complicates studies of carbon monoxide inhibition on these animals.



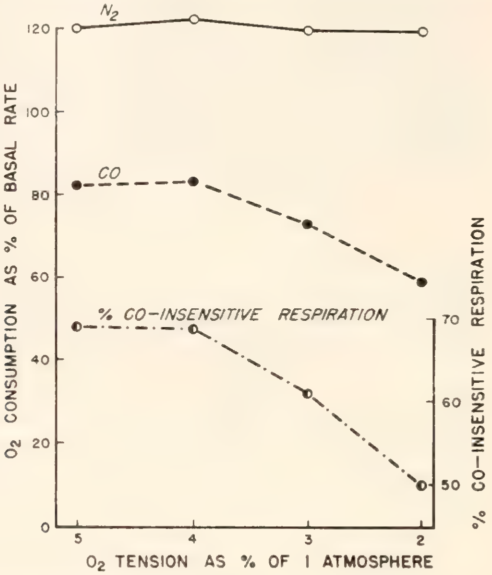


FIGURE 1 (left). The CO-insensitivity of pupal respiration as a function of basal O_2 consumption. CO/O_2 ratio = 19:1. The oxygen tension was 5% in both CO and N_2 mixtures. The gas exchange of 18 diapausing Cynthia pupae was measured first in the N_2 mixture and then in the CO mixture. The per cent of CO-insensitive respiration is plotted against the respiration in the N_2 mixture.

FIGURE 2 (right). The CO-sensitivity of pupal respiration at reduced O_2 tensions. The average O_2 consumption of four brainless Promethea pupae whose average O_2 consumption in air was 33 mm.^{3}/gm. live wt./hr. is recorded at each successive O_2 tension in the O_2-N_2 mixtures. Similarly the average O_2 consumption of five other pupae, whose average O_2 consumption in air was 27 mm.^{3}/gm. live wt./hr., is recorded at each O_2 tension in the O_2-CO-N_2 mixtures. The rate of respiration in each gas mixture is expressed as per cent of basal rate in air. The CO/O_2 ratio was kept constant at 19:1 by adding appropriate amounts of N_2 to the O_2-CO mixture. The right-hand vertical axis records the per cent of CO-insensitive respiration. Oxygen consumption decreased at tensions below 2% and this so complicated measurements of CO inhibition that values for O_2 tensions below 2% could not be calculated. The average weight of the pupae was 2 grams.

C. *The CO-sensitivity of pupal respiration at reduced oxygen tensions*

In the following experiment the CO-sensitivity of the respiration of a group of Promethea pupae was measured at oxygen tensions ranging from 5 to 2 per cent of an atmosphere. The results summarized in the lower curve in Figure 2 disclose that at low oxygen tensions pupal respiration becomes sensitive to carbon monoxide. As the figure reveals, pupal respiration was not depressed by low oxygen alone down to 2 per cent. In sharp contrast to this insensitivity of respiration to low oxygen tensions in the nitrogen-oxygen mixtures, the respiration in 19:1 CO/O_2 remained constant only between 5 and 4 per cent oxygen and then progressively decreased as the oxygen tension decreased. In other words, at low oxygen tensions pupal respiration is inhibited by carbon monoxide. These observations suggest that cytochrome oxidase is functioning at all times in the diapausing pupa but at higher oxygen tensions its CO-sensitivity is in some manner masked. A similar experiment performed on Cynthia pupae yield substantially the same results.

The cause of the slight stimulation of respiration shown in the figure in nitrogen-oxygen mixtures containing 2 per cent oxygen or more is unknown. In 1 per cent oxygen respiration fell to about 85 per cent of the basal rate. It is significant that these measurements were conducted on Promethea pupae less than one half the size of Cecropia pupae used by Schneiderman and Williams (1954a) in an experiment appraising the effect of oxygen tension on pupal respiration. They reported that the respiration of Cecropia pupae decreased when oxygen tension fell below 5 per cent. These contrasting results are explained by the fact that in the larger Cecropia pupae the diffusion distances are greater than in Promethea pupae. Therefore, the actual tension of oxygen within the pupal tissues is probably less for large pupae than small ones. As a result, the respiration of large pupae is limited at oxygen tensions which do not affect the respiration of small pupae.

D. *The effects of sodium azide and antimycin A on pupal respiration*

Three groups of five diapausing Cynthia pupae, whose average basal respiration was 15.4 $mm.^3/gm.$ live wt./hr., were injected with sodium azide to internal concentrations of 10^{-5} *M*, 10^{-4} *M*, and 5×10^{-4} *M*. The average respiration of these pupae on the day of injection was indistinguishable from the respiration of five control pupae injected with water. Since azide is an extremely soluble small molecule, it is doubtful that impermeability is responsible for this insensitivity. Pupal respiration is thus relatively insensitive to azide as well as to carbon monoxide.

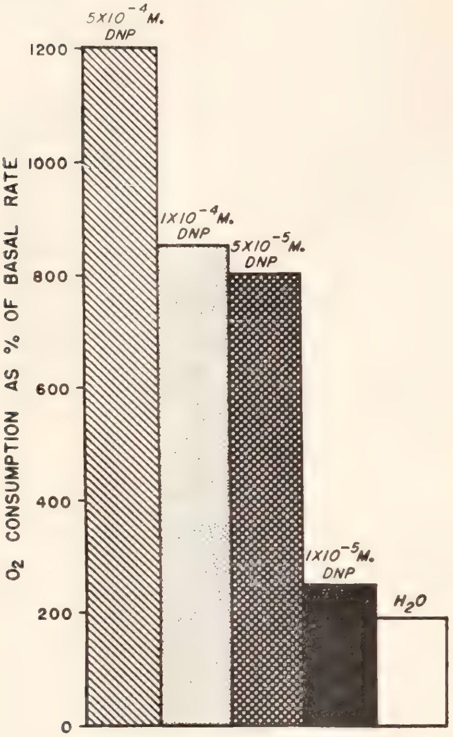
In a similar experiment, the effects of antimycin A on respiration were examined in fifteen Cynthia pupae whose average basal respiration was 13.3 $mm.^3/gm.$ live wt./hr. A control group of five received a 1 per cent ethyl alcohol solution, a second group received antimycin A to an internal concentration of 10^{-7} *M* and the third group received antimycin A to a final concentration of 10^{-6} *M*. Injecting the 1 per cent ethyl alcohol had exactly the same effect as injecting distilled water and promptly doubled the respiration. Compared with the ethyl alchol control, 10^{-7} antimycin A inhibited respiration about 20 per cent and 10^{-6} *M* about 30 per cent. Similar concentrations of antimycin A commonly cause much higher inhibitions in other organisms and the respiration of the diapausing pupa may be considered relatively insensitive to this potent inhibitor of the cytochrome *c* reductase system.

2. *DNP-stimulated respiration*

A. *The stimulatory effect of DNP*

An important clue to the nature of the oxidative pathways of diapausing pupae was uncovered in 1955 by Harvey and Shappirio (unpublished observations) who discovered that DNP stimulated the respiration of diapausing Cecropia pupae and that this respiration was CO-sensitive. This result, which they generously shared with us, agreed with the earlier observations of Bodine and Boell noted in the Introduction, and suggested to us a number of experiments using DNP.

A series of Cynthia pupae were injected with DNP to internal concentrations ranging from 5×10^{-4} *M* to 10^{-5} *M*. Figure 3 records the average initial stimulation of respiration of several concentrations for the first three hours after injection, while Figure 4 records the respiratory behavior of the 10^{-4} *M* group over a five-day period. Dinitrophenol called forth an immediate and spectacular increase in oxygen consumption which averaged 12 times the basal rate in the case of pupae receiving 5×10^{-4} *M*. As Figure 4 shows, in the group receiving 10^{-4} *M* the initial acceleration of respiration on the day of injection was followed by a decline on the following



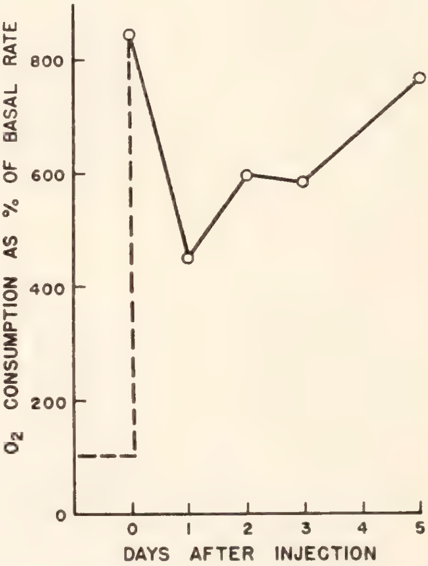


FIGURE 3 (left). The effect of several concentrations of DNP on the respiration of diapausing pupae. Five Cynthia pupae received injections of DNP to an internal concentration of 5×10^{-4} *M*, four received 10^{-4} *M*, four received 5×10^{-5} *M*, four received 10^{-5} *M*, and a control group of four received distilled water. The average O_2 consumption for the first three hours after injection is recorded.

FIGURE 4 (right). The effect of DNP injection on the O_2 consumption of diapausing pupae. Four Cynthia pupae whose average O_2 consumption was 13.0 mm.^{3}/gm. live wt./hr. were injected with DNP to an internal concentration of 1×10^{-4} *M*. Respiration was measured for three hours each day over a five-day period. The day of injection is denoted as day "0".

day. This was succeeded by a gradual increase of respiration over a three-day period, to a peak on the fifth day after injection almost as great as the initial peak respiration. The respiration returned to about normal approximately two weeks later. The initial stimulation of respiration is doubtless due to the uncoupling effect of DNP which causes an acceleration of the turnover rate of the components of the respiratory chain (Cross *et al.*, 1949; Chance and Williams, 1956). The secondary effects which develop several days later, appear to be the result of (a) injury-stimulated respiration provoked by injection through the cuticle (see Section 3 below) and (b) the development of an "energy debt" metabolism (analogous to an "oxygen debt repayment" (Kurland *et al.*, 1958)) as the result of prolonged uncoupling of phosphorylation by DNP. Comparable results were obtained with diapausing pupae of Cecropia, Promethea and Polyphemus.

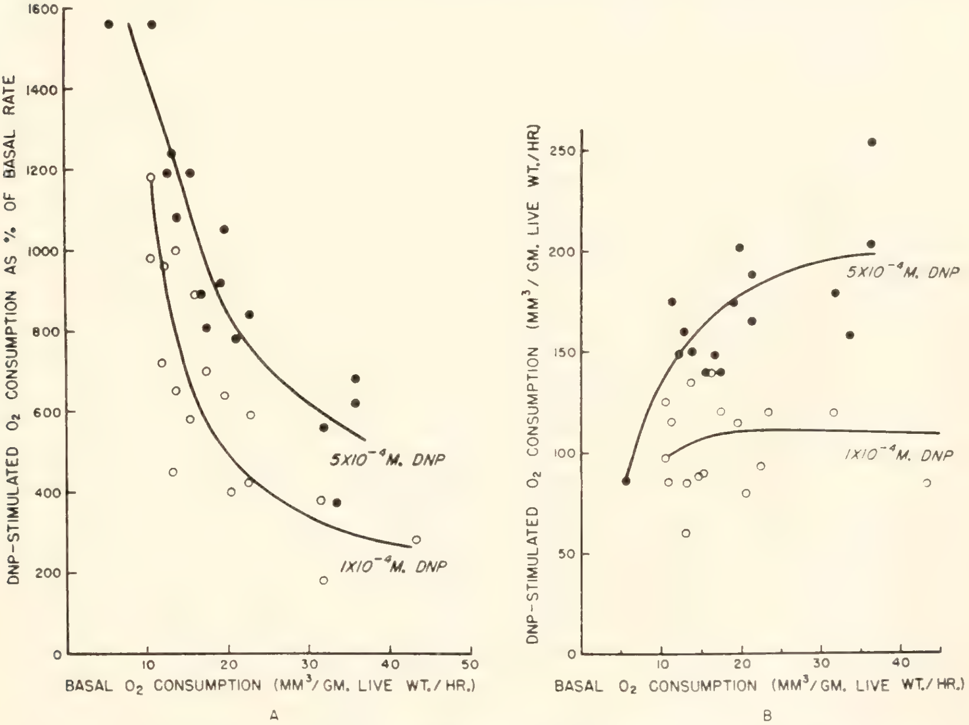


FIGURE 5. The stimulation of O_2 consumption by DNP as a function of basal respiratory rate. (A) The percentage stimulation of O_2 consumption of Cynthia pupae after injection of DNP to internal concentrations of 5×10^{-4} *M* and 10^{-4} *M* is plotted as a function of basal respiration. (B) The total DNP-stimulated respiration of the pupae in (A) is plotted as a function of the basal respiration.

The time course of the respiratory changes recorded in Figure 4 is also typical of pupae receiving 5×10^{-4} *M* DNP but the pattern differed somewhat in pupae that received lower concentrations. Because the initial stimulation of respiration was less, the fall in respiration recorded in Figure 4 was commonly absent. The

5×10^{-4} *M* concentration is apparently close to the lethal level and occasional individuals died about a week after receiving that amount.

Further analysis of the effects of DNP disclosed that pupae with high initial basal respirations were proportionately less stimulated by DNP than were pupae with low basal metabolic rates. Figure 5A shows that a Cynthia pupa with a basal metabolic rate of 5 mm.^{3}/gm. live wt./hr. experienced a 16-fold stimulation of respiration after injection of DNP whereas a similar pupa with a basal respiration of 30 mm.^{3}/gm. live wt./hr. experienced only a 6-fold stimulation of respiration. Thus, there is a steep decline in the per cent of DNP-stimulated respiration as basal respiration increases. Figure 5B further reveals that DNP-stimulated respiration approaches a limit as the basal respiration approaches 25 mm.^{3}/gm. live wt./hr. The significance of this limit will be considered in the Discussion.

B. *The effect of carbon monoxide, azide and antimycin A on DNP-stimulated respiration*

Diapausing Cynthia pupae were injected with DNP and then exposed to carbon monoxide. The results, summarized in Figure 6, reveal that about half the DNP-stimulated respiration was inhibited by carbon monoxide. Further analysis of the data from this experiment revealed that CO-sensitivity increased slightly as the rate of oxygen consumption increased. Thus pupae with a DNP-stimulated respiration of 90 mm.^{3}/gm. live wt./hr. had only 45 per cent of their respiration inhibited by carbon monoxide, whereas pupae with a DNP-stimulated respiration of 125 mm.^{3}/gm. live wt./hr. had nearly 70 per cent of their respiration inhibited by carbon monoxide.

The effect of azide on DNP-stimulated respiration of diapausing Cynthia pupae is recorded in Figure 7. There was no significant initial inhibition of the respiration when sodium azide alone was injected (see Section 1D), but some of the DNP-stimulated respiration was inhibited by this reagent. Indeed, as Figure 7B shows, more than three-fourths of the DNP-stimulated respiration was inhibited by 5×10^{-4} *M* sodium azide. However, in group B only half of the pupae receiving injections of DNP and none of the pupae receiving sodium azide survived for more than a week, indicating these high concentrations of antimetabolites were ultimately toxic. Comparable results were obtained with Cecropia pupae.

Experiments appraising the antimycin A-sensitivity of DNP-stimulated respiration were conducted on a series of 15 Cynthia pupae which received 10^{-4} *M* DNP and 10^{-6} *M* antimycin A. About 30 per cent of the DNP-stimulated respiration was inhibited by this concentration of inhibitor. Thus, the respiration of DNP-stimulated pupae is no more sensitive to antimycin A than the respiration of normal pupae.

3. *Injury-stimulated respiration*

A. *The CO-sensitivity of injury-stimulated respiration*

As mentioned previously, integumentary injuries to pupae dramatically accelerate respiration for one to three weeks (Schneiderman and Williams, 1953a, 1953b). Moreover, this accelerated respiration is proportional to the extent of injury and seems to be caused in part by diffusible substances released at the site of injury (Jankowitz, 1955; Schneiderman, 1957). Although the respiration induced by a small incision into Cecropia pupae was not inhibited by carbon monoxide, repair of extensive wounds was prevented by this gas (Schneiderman and Williams, 1953b, 1954b), suggesting that the cytochrome oxidase system was functioning in injured pupae.

To investigate this possibility the following experiments were carried out. Four Cynthia pupae were given a large injury by removing their pupal legs; two pupae were immediately placed in 7 per cent oxygen and nitrogen, and the other two were placed in a corresponding atmosphere of oxygen and carbon monoxide. The pupae were maintained in their respective gas mixtures for one week, and the gas mixtures were renewed thrice daily.

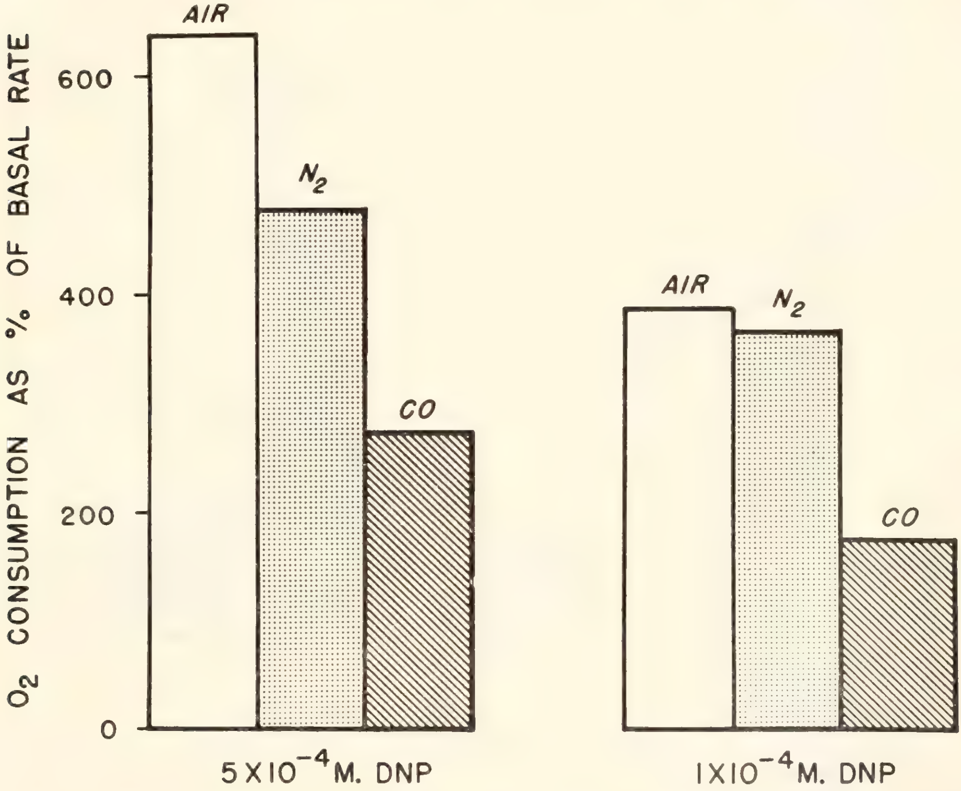


FIGURE 6. The CO-sensitivity of DNP-stimulated respiration. Two groups of five diapausing Cynthia pupae were injected with DNP to internal concentrations of 5×10^{-4} *M* and 10^{-4} *M*. All the pupae were exposed to 5% O_2 and 95% N_2 and then to 5% O_2 and 95% CO ($CO/O_2 = 19:1$). The average respiration over a one-hour period is recorded.

As Figure 8 shows, injured pupae in the nitrogen mixture developed a characteristic injury respiration; on the other hand, those in carbon monoxide mixtures did not. Indeed, five days after injury both of the pupae maintained in carbon monoxide had died. Thus carbon monoxide apparently caused death by preventing the development of injury respiration. Similar results were obtained with Promethea pupae. These results are in general agreement with those of Schneiderman and Williams (1954b), who reported that the repair of injury was CO-sensitive. However, their experiments failed to detect the CO-sensitivity of the respiration associated with repair of injury, presumably because they employed only small injuries. Such CO-sensitivity was demonstrated by Harvey and Shappirio (Harvey, 1956) who pointed out that after very large injuries respiration becomes sensitive to carbon monoxide. This is confirmed in the following experiment summarized in

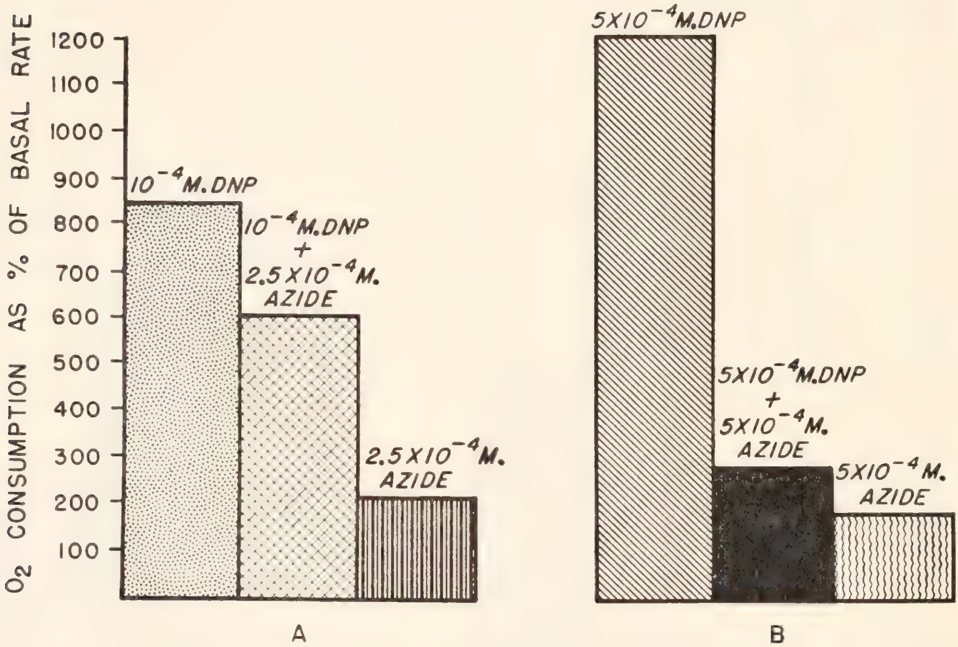
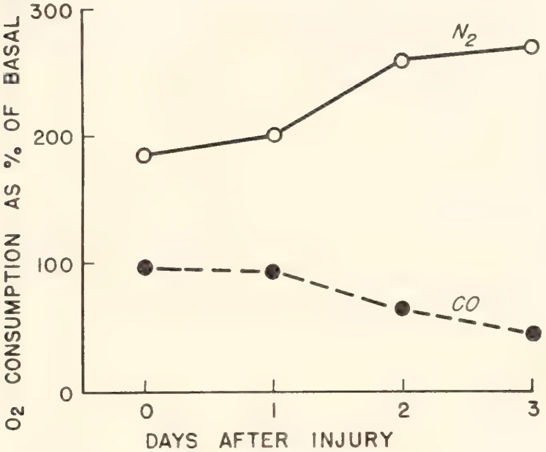


FIGURE 7. The azide-sensitivity of DNP-stimulated respiration. (A) Four diapausing Cynthia pupae were injected with 10^{-4} *M* DNP, four with 2.5 *M* sodium azide, and four with both reagents. The average O_2 consumption over a three-hour period is recorded. (B) Five pupae were injected with 5×10^{-4} *M* DNP, five with 5×10^{-4} *M* sodium azide and five with both reagents. The average O_2 consumption over a three-hour period is recorded. The average initial oxygen consumption of the pupae in (A) and (B) was 16.8 mm.^{3}/gm. live wt./hr.

Figure 9. Four brainless Cynthia pupae were injured by removing the pupal legs and after three days, when they had developed a large injury respiration, the CO-sensitivity of their respiration was determined. About two-thirds of the injury respiration was inhibited by carbon monoxide. Similar results were obtained with Cecropia pupae. It can also be seen in Figure 9 (as well as in Figure 6) that the oxygen uptake of pupae respiring at a rapid rate was limited by the low oxygen tension. This contrasts with the respiratory behavior of pupae with low metabolic rates, where 5 per cent oxygen and 95 per cent nitrogen commonly stimulated oxygen consumption (see Section 1C).



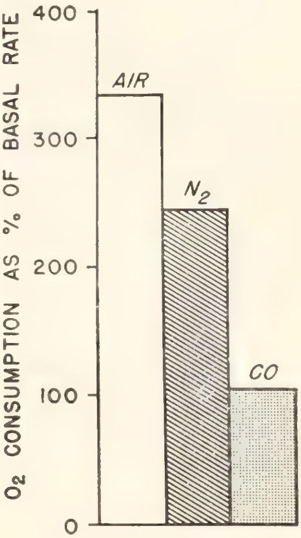
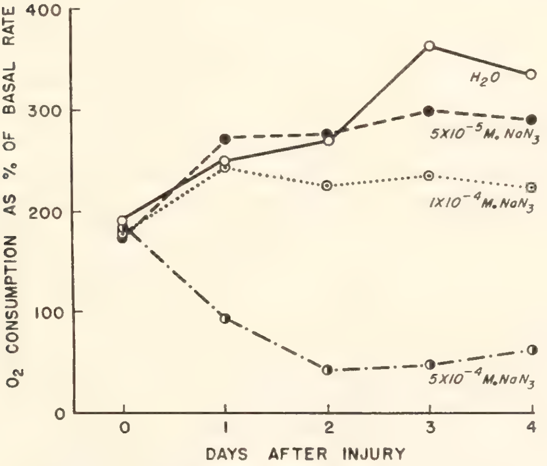


FIGURE 8 (left). The effect of injury and simultaneous exposure to CO on respiration. Two injured Cynthia pupae were maintained continuously in 7% O_2 plus N_2 and two were maintained in 7% O_2 plus CO ($CO/O_2 = 13:1$). The average respiration of each pair over a 3-hour period is recorded as a function of time. The day of injury is denoted as day "0".

FIGURE 9 (right). The CO-sensitivity of injury respiration. The average respiration over a 4-hour period of four brainless Cynthia pupae 3 days after injury in air, in 5% O_2 and 95% N_2, and in 5% O_2 and 95% CO (CO/O_2 ratio = 19 : 1).



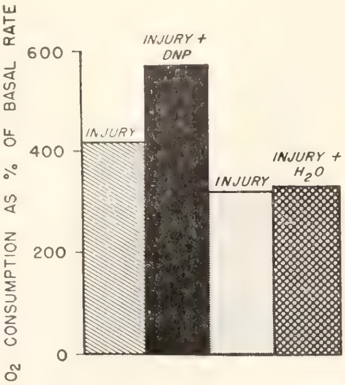


FIGURE 10 (left). The effect of azide injection on the O_2 consumption of four groups of five diapausing Cynthia pupae over a five-day period. The day of injection is denoted as day "0".

FIGURE 11 (right). The effect of DNP on injury respiration. The average O_2 consumption over a 3-hour period of two injured diapausing Cynthia pupae prior to and after the injection of water, and of two injured pupae prior to and after the injection of 5×10^{-4} *M* DNP.

B. *Injury-stimulated respiration in newly pupated Cecropia*

Four Cecropia silkworms were injured within one day after pupation by removing a rectangular window of pupal cuticle from their faces. No significant stimulation of respiration was observed. Since injury respiration is characteristic of diapausing pupae, and since the respiration of newly pupated Cecropia is much greater than the respiration of pupae firmly in diapause, this result suggests that the production of injury respiration is intimately associated with the extremely low respiration of the diapausing insect (see Discussion).

C. *The azide-sensitivity of injury-stimulated respiration*

The effect of azide on injury respiration was examined by injecting a series of diapausing Cynthia pupae with sodium azide at several concentrations. In this experiment the injection itself served as the injury. The average daily respiration of each group of pupae over a five-day period is plotted in Figure 10.

All pupae treated with 5×10^{-4} *M* sodium azide died within 10 days after injection, indicating either (a) that this concentration had a simple toxic effect, or (b) that the development of injury-stimulated respiration was inhibited by azide and this caused death, as was the case when injured pupae were exposed continuously to mixtures of carbon monoxide and oxygen (see Section 3A above). In lower concentrations of azide, the inhibition of injury respiration was proportional to concentration.

TABLE II

Effect of simultaneous injury and injection of DNP on the respiration of six diapausing Cynthia pupae

Basal respiration, mm.^{3}/gm./hr.	Treatment	Max. resp. as % basal rate	Day of max.
25.0	Injury + H_2O	258	2
12.0	Injury + H_2O	521	2
31.0	Injury + H_2O	234	3
26.5	Injury + DNP	449	6
40.5	Injury + DNP	496	6
30.5	Injury + DNP	Died	–

D. *The effects of DNP on injury-stimulated respiration*

The pupal legs were removed from a group of four Cynthia pupae. Three days after wounding, when injury respiration had reached its maximum, two of the pupae received injections of DNP to an internal concentration of 5×10^{-4} *M*, and the remaining two received injections of water. The data summarized in Figure 11 show that 5×10^{-4} *M* DNP caused a significant acceleration of maximum injury respiration; however, the increase was proportionately much less than that encountered in DNP-treated diapausing pupae. Comparable results were obtained with Cecropia and Promethea pupae.

In another experiment, six diapausing pupae were injured by removing their pupal legs; half these pupae immediately received water injections, while the remainder received injections of DNP to an internal concentration of 5×10^{-4} *M*. The respiration of these pupae is summarized in Table II. The maximum respiration of injured pupae treated with DNP was reached six days after the injury, while those receiving injections of water displayed maximum respiration two or three days after injury. Thus DNP delayed the development of injury respiration.

Discussion

1. *A new explanation for the insensitivity of pupal respiration to carbon monoxide*

Studies noted in the Introduction have shown that the onset of pupal diapause in giant silkworms is accompanied by a precipitous fall in the rate of oxygen consumption, and that the low respiration of the diapausing pupa is virtually uninhibited by carbon monoxide and cyanide. As judged by its insensitivity to these inhibitors, nearly all of the respiration of the diapausing pupa appeared to proceed *via* pathways independent of cytochrome oxidase. Hence it was suggested that the respiration of the diapausing pupa was mediated by a terminal oxidase other than cytochrome oxidase, possibly a flavoprotein or an autoxidizable cytochrome of the *b* type (Schneiderman and Williams, 1954a, 1954b). This suggestion was taken up by various investigators (Cotty, 1956; Ito, 1955). The present experiments provide an alternative explanation for the CO-insensitivity of pupal respiration; namely, that it is due to a great excess of cytochrome oxidase relative to trace amounts of cytochrome *c* in most of the tissues of the diapausing pupa. This limitation of cytochrome *c* leads to an unsaturation of cytochrome oxidase, and this in turn leads to the insensitivity of pupal respiration to carbon monoxide and azide. Under this view the principal factor underlying the *low respiration* of the diapausing pupa is the *limitation of cytochrome c* in most of the pupal tissues, while the principal factor underlying the *CO- and azide-insensitivity* of pupal respiration is the *excess of cytochrome c oxidase* in most of the pupal tissues. Thus, quantitative changes in the relative amounts of respiratory enzymes after pupation are responsible for both the low over-all respiration of diapause and for CO-insensitivity. In other words, the basic differences between the respiratory enzyme systems of diapausing and non-diapausing insects are *quantitative,* but they lead to *qualitative* differences in the response of the insect to certain inhibitors. Contrary to earlier opinions, cytochrome oxidase appears to be the principal terminal oxidase during diapause as well as during all the other stages of the life history.

2. *Preliminary theoretical considerations*

It can be shown that an excess of cytochrome oxidase may lead to a virtual CO-insensitivity of respiration that is actually mediated by cytochrome oxidase, and in the final section of this discussion a brief theoretical analysis of this assertion is presented. The argument offered is that when cytochrome oxidase is in great excess and thus not saturated, a large fraction of the cytochrome oxidase may be inhibited by carbon monoxide without affecting the rate of electron transfer from cytochrome *c*. Stated in another way the greater the "saturation" of cytochrome oxidase by cytochrome *c*, the greater the CO-sensitivity of respiration; the less the "saturation" of cytochrome oxidase by cytochrome *c*, the less the CO-sensitivity. This conclusion seems intuitively acceptable and is proven in Section 9 (below). Recognizing this relation between CO-sensitivity and saturation of cytochrome oxidase it is not difficult to interpret the several experimental results.

3. *Carbon monoxide experiments*

Evidence presented previously has shown that the specific target of carbon monoxide in the insect at all stages is reduced cytochrome *c* oxidase (Schneiderman and Williams, 1954a, 1954b). A principal factor determining the impact of carbon monoxide on cytochrome oxidase is the CO/O_2 ratio: the higher this ratio, the greater the proportion of reduced cytochrome oxidase molecules inhibited. The CO/O_2 ratios employed in the present experiments were usually 16:1 or 19:1 and ambient oxygen tensions were maintained at 6 or 5 per cent. Direct analysis of the composition of the tracheal gas of normal diapausing pupae kept at these oxygen tensions by a precise microgasometric method (Levy and Schneiderman, 1957, 1958) revealed that the actual oxygen tension within the tracheal system, and hence within the insects' tissues, was about 1 per cent lower than ambient, that is, about 5 or 4 per cent. Therefore in the present experiments the actual CO/O_2 ratios within the pupal tissues approached 24:1. Since it has been shown that a CO/O_2 ratio of 16:1 causes a 50 per cent light-reversible inhibition of the cytochrome oxidase activity of homogenates of the thoracic muscles of Cecropia moths (Pappenheimer and Schneiderman, unpublished), we may conclude that *the CO/O_2 ratios used in the present experiments were capable of inhibiting no less than 50 and probably as much as 75 per cent of the reduced cytochrome oxidase activity of homogenates of the insect's tissues.* However, as we have already noted in the previous section, the inhibition in a homogenate where cytochrome oxidase is saturated by added cytochrome *c* may be quite different from the inhibition observed in the intact insect where the cytochrome oxidase may not be saturated by cytochrome *c*. Let us now consider what our several experiments tell us about the saturation of cytochrome oxidase in the diapausing pupa.

Perhaps the most crucial result is recorded in Figure 2. As the figure shows, when the oxygen tension is reduced to 2 per cent in a mixture of oxygen and nitrogen, the oxygen consumption of the pupa remains about the same as in air, but the CO-sensitivity of the respiration is enhanced. The simplest interpretation of this result is that cytochrome oxidase is present in excess over some rate-limiting link in the respiratory chain, and only at low oxygen tensions does the cytochrome oxidase-oxygen reaction become the limiting step in the respiratory chain, subject, as a consequence, to inhibition by carbon monoxide.

The reasons for stimulatory effects of carbon monoxide on pupae with low metabolic rates (*cf.* Fig. 1) are not yet clear. Similar stimulatory effects of carbon monoxide have been reported by Bodine and Boell (1934a) for *Melanoplus,* by Klein and Runnström (1940) for unfertilized eggs of the sea urchin, and by others (*cf.* review by Needham, 1942, p. 496). Possibly it does not represent stimulation of respiration but is simply gas uptake due to an actual oxidation of CO by the tissues to CO_2 (*cf.* review of Lilienthal, 1950). Perhaps it is something different altogether, such as an uncoupling action (Thimann *et al.,* 1954). For our present purposes suffice it to say that the phenomenon, although not yet explained, does not affect our interpretation of the basic action of carbon monoxide on cytochrome oxidase and the argument that cytochrome oxidase is only partially saturated in pupal tissues. Further evidence supporting this argument derives from studies with DNP and azide which are considered in Sections 4 and 5 below.

Significant data revealing the degree of saturation of cytochrome oxidase in the diapausing pupa are also to be found in the observation that CO-sensitivity of pupal respiration increases with increasing basal respiration and is instantly enhanced by DNP, and in the fact that the increased respiration that follows injury or the initiation of adult development is inhibited by carbon monoxide. Moreover, we have found that the increased respiration that follows a prolonged period of anoxia is also sensitive to carbon monoxide. These results, which are summarized in Table III, lead to the conclusion that *the fraction of respiration sensitive to carbon monoxide is a function of the rate of oxygen consumption of the silkworm at all stages.* This implies that virtually any process which increases the rate of pupal respiration increases the saturation of cytochrome oxidase and that, in the pupa, cytochrome oxidase is in great excess and hence very unsaturated.

TABLE III

Summary of the effects of metabolic inhibitors on the respiration of diapausing pupae in various physiological states and on developing adults

	Effect			
Inhibitor / Physiological condition	CO CO O_2 = about 20:1	DNP	Azide	Antimycin A
Diapause respiration	Stimulation at low basal rates Slight or no inhibition at modest basal rates. Inhibition increases as basal respiration increases Up to 50% inhibition at low oxygen tensions	5×10^{-4} *M* stimulates respiration an average of 12-fold and as much as 16-fold Stimulation less at high basal rates	No immediate effect at concentrations up to 5×10^{-4} *M*	30% inhibition at 10^{-6} *M*
DNP stimulated respiration	An average of 50% inhibition	--	30 to 70% inhibition depending on concentration of azide	30% inhibition at 10^{-6} *M*
Injury-stimulated respiration	No or slight inhibition after small injury; up to 60 per cent inhibition after large injury Exposure immediately after injury prevents development of injury-stimulated respiration	Stimulation by DNP inversely proportional to size of injury-stimulated respiration. After large injuries, about 2-fold stimulations by DNP. Injection of DNP immediately after injury delays development of injury respiration	Inhibition proportional to concentration of azide	-
Developing adult	More than 50% inhibition (Schneiderman and Williams, 1954a)	5×10^{-4} *M* stimulates respiration about 2-fold	-	-

Recognizing the importance of low over-all respiratory rate as a factor in CO-insensitivity, it is worthwhile considering certain diapausing insects whose respiration is not resistant to carbon monoxide or cyanide. Two species whose respiration continues to be inhibited by carbon monoxide or cyanide during diapause are prepupae of the larch sawfly, *Pristophora* (McDonald and Brown, 1952), and larvae of the horse bot fly, *Gastrophilus* (Levenbook, 1951). It is of considerable significance that the respiration of these insects at 25° C. is many times greater than the respiration of diapausing silkworm pupae. Thus the respiratory rate of *Pristophora* is about 165 mm.^{3}/gm. live wt./hr., while that of *Gastrophilus* is more than 100 mm.^{3}/gm. live wt./hr.[3] This compares with a respiratory rate for diapausing silkworm pupae of 8 to 20 mm.^{3}/gm. live wt./hr. Furthermore, in diapausing silkworm pupae only the skeletal muscles, of which there are few, have a saturated cytochrome *c* oxidase, and these account for only a small fraction of the insect's total respiration. In diapausing species with high respiratory rates like *Pristophora* and *Gastrophilus* it appears likely that (1) they have more muscular tissue and this accounts for a larger fraction of their total respiration than do the muscles of diapausing pupae, and (2) some of their non-muscular tissues may have a saturated cytochrome oxidase. These factors could easily account for their sensitivity to carbon monoxide.

4. *The significance of DNP-stimulated respiration*

The experiments with DNP demonstrate that in diapausing pupae cytochrome oxidase is not fully saturated. As is well known, DNP increases the turnover of the respiratory carriers, presumably because it is able to uncouple phosphorylation from electron transfer, and so increases the demand for oxygen (Chance and Williams, 1956). The data in Section 2 reveal a striking 12- to 16-fold acceleration of pupal oxygen consumption by 5×10^{-4} *M* DNP. This may be one of the largest DNP stimulations ever recorded. It contrasts with the finding of Bodine and Boell (1938) that the respiration of diapausing *Melanoplus* eggs was accelerated a maximum of only 3.5 times by 3×10^{-5} *M* DNP, while further increase in concentration produced a submaximal response. De Meio and Barron (1934) and Maroney *et al.* (1957) have reported DNP stimulations in various invertebrate tissues of only about two-fold. Aside from the magnitude of DNP-stimulated respiration (which by itself suggests unsaturation of cytochrome oxidase), the CO-sensitivity of DNP-stimulated respiration is of special interest. It indicates that DNP accelerates the turnover of several carriers of the respiratory chain but has a lesser effect on the turnover of cytochrome oxidase. This conclusion arises from the fact that CO-sensitivity is a function of the saturation of cytochrome oxidase. The CO-sensitivity of DNP-stimulated respiration tells us that DNP increases the saturation of cytochrome oxidase. Thence it follows that DNP not only accelerates over-all respiratory rate but alters the quantitative relationship between cytochrome oxidase and the intermediate carriers in the respiratory chain.

One of these accelerated carriers is almost certainly cytochrome *c*, which may be the most important rate-limiting carrier in the respiratory chain, a point we shall consider further in Section 6 (below). Dinitrophenol appears to increase in some way the effective turnover of this enzyme and increases thereby the saturation of cytochrome oxidase. It is significant that both the absolute magnitude and the CO-sensitivity of DNP-stimulated respiration were lower for pupae with low basal respiration. Thus in the present experiments, although pupae with low basal metabolic rates were proportionately more stimulated by DNP than pupae with high basal metabolic rates, the latter developed a greater over-all respiration under the influence of DNP. The data also reveal that CO-sensitivity reached a maximum of about 70 per cent when DNP-stimulated respiration reached its maximum. We interpret these findings to mean that pupae with low basal respiration have less cytochrome *c* available to be turned over and, as a result, these pupae are not capable, even under the influence of high concentrations of DNP, of completely saturating their cytochrome *c* oxidase and thereby achieving maximum CO-sensitivity. These DNP studies provide support for the argument that the low over-all respiration of diapause is due to a low concentration of some respiratory component, probably cytochrome *c*, whereas the CO-insensitivity is the result of the relatively high concentration of cytochrome *c* oxidase.

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[3] This last value was calculated from values obtained at 37° C. by assuming a Q_{10} of about 2.5.

In our experience the respiration of developing adults is accelerated by DNP to a much lesser extent than that of diapausing pupae, usually about two-fold. This fact suggests that in the developing adult, as contrasted with the diapausing pupa, cytochrome oxidase is virtually saturated. Also, although development may be delayed, developing adults survive concentrations of DNP which are toxic to diapausing pupae, possibly because their higher metabolic rate enables them to metabolize the DNP (*cf.* Cross *et al.*, 1949).

5. *The significance of azide-insensitive respiration*

In these insects it seems safe to identify cytochrome oxidase as the main target of azide (Horecker and Stannard, 1948; Stannard and Horecker, 1948). The experiments summarized in Section 1D disclosed that azide had no immediate effect on diapause respiration at concentrations as high as 5×10^{-4} *M*. This result supports the conclusion drawn above, that cytochrome oxidase does not limit pupal respiration. On the other hand, the sensitivity to azide of DNP-stimulated respiration was quite striking. This is consistent with the argument that, under the influence of DNP, cytochrome oxidase becomes more saturated.

6. *The limiting link in the pupal respiratory chain*

The present experiments provide only one clue to the identity of the limiting link in the pupal respiratory chain. This is the fact that antimycin A—a potent inhibitor at the concentrations we employed of the DPNH-cytochrome *c* reductase system—had only a minor effect on normal pupal respiration and DNP-stimulated respiration. This inhibitor is said to have as its specific target the Slater factor which mediates the transfer of electrons from flavoprotein to cytochrome *c* (Potter and Reif, 1952; Reif and Potter, 1953; Chance and Williams, 1956). The insensitivity of pupal respiration to this reagent suggests that the limiting link in the pupal respiratory chain lies between the Slater factor and cytochrome oxidase, *e.g.*, cytochrome *c*. Recent studies of Shappirio and Williams (1957a, 1957b) indicate that the limiting link is very likely cytochrome *c*, for with very sensitive spectroscopic techniques they were unable to detect this enzyme in most pupal tissues although cytochrome oxidase was easily demonstrated. They also showed that in homogenates of pupal tissues, cytochrome *c* is a rate-limiting link in the oxidation of DPNH. Hence it seems safe to identify limiting concentrations of cytochrome *c* as a principal cause of the unsaturation of cytochrome oxidase in pupal tissues.

7. *Injury-stimulated respiration*

The increased sensitivity to carbon monoxide and azide shown by pupae supporting an injury respiration (Sections 3A and 3C) indicate an increased saturation of cytochrome oxidase after injury. The observation (Section 3D) that 5×10^{-4} *M* DNP failed to accelerate injury respiration to the same degree as diapause respiration supports the conclusion that cytochrome oxidase is virtually saturated when injury respiration is at its maximum. What brings about this increased saturation of cytochrome oxidase is not known with certainty but the present experiments suggest that it is caused by a gradual synthesis of cytochrome *c* which is provoked by injury. Recall that injured pupae treated with DNP were delayed in developing maximum injury respiration when compared with injured pupae receiving water injections. This suggests that integumentary injury initiates some process which requires a supply of phosphate bond energy which was uncoupled by DNP. The gradual development of maximum injury respiration over a three-day period suggests further that this energy-demanding process involves, in part, the synthesis of one or more of the respiratory chain components and does not simply reflect increased turnover of pre-existing enzymes. We interpret the increased CO-sensitivity of injury-stimulated respiration to indicate that more cytochrome *c* is being synthesized than cytochrome oxidase. That augmented protein synthesis does in fact follow injury has been demonstrated by Telfer and Williams (1955), who showed that the incorporation of C^{14}-labelled glycine into the pupal proteins was stimulated by injury to about the same extent as respiration.

It is not without interest that the synthesis of these respiratory components appears to be obligatory. Indeed, the data in Section 3A suggest that when synthesis is prevented by prolonged exposure to carbon monoxide, the pupae fail to develop an injury respiration and die. This obligatory synthesis of new respiratory components may be imposed upon diapausing pupae because their capacity for wound repair is restricted by their low metabolic rate. Apparently this repair process is able to compete with the "maintenance" processes of the diapausing pupa, thereby causing death when total energy production is reduced by carbon monoxide. In this connection, it is noteworthy that newly molted pupae, whose respiratory rate is considerably larger than that of pupae firmly in diapause, fail to show an injury respiration. This reflects their capacity to underwrite the energy requirements of injury without augmenting the respiratory chain. This capacity is also present in developing Cecropia adults and we have also shown it in all stages of non-diapausing species such as the bee-moth *Galleria mellonella*.

8. *Conclusions*

The several lines of evidence considered in the preceding sections persuade us that earlier conceptions of the respiratory enzyme system of diapausing silkworms need re-evaluation. The basic differences between the respiratory enzyme chains of the diapausing pupa and the non-diapausing stages appear to be *quantitative* differences and not *qualitative* differences as was suggested earlier (Williams, 1951; Schneiderman and Williams, 1954a, 1954b). The CO-insensitivity of pupal respiration does not stem from the activity of a CO-insensitive terminal oxidase, but results from a great excess of cytochrome oxidase relative to other components of the respiratory chain. None of our findings supports the renewed suggestions of Wojtczak (1955) and Ito (1955) that tyrosinase functions as a terminal oxidase in insects. Indeed, in view of the failure of potent inhibitors of tyrosinase like phenylthiourea to inhibit respiration (Schneiderman and Williams, 1954a) and the light-reversibility of the carbon monoxide inhibition of silkworm growth (Schneiderman and Williams, 1954b) and respiration (Pappenheimer and Schneiderman, unpublished) this is not likely. The present data, coupled with the recent spectroscopic findings of Shappirio and Williams (1957a, 1957b) and with the studies of Harvey and Williams (1958a, 1958b) on the pupal heart, indicate that cytochrome oxidase is the terminal oxidase during pupal diapause and cytochrome *c* is the limiting component in the pupal respiratory chain.

In this perspective, the increased respiration following integumentary injury and initiation of adult development reflects an increase in cytochrome *c* content which occurs at a faster rate than any increase in cytochrome oxidase. Possibly the increase in cytochrome *c* reflects its adaptive synthesis in response to changes in the energy requirements of the tissues. These changes were induced on the one hand by injury and on the other by the prothoracic gland hormone which initiated adult development. Such an adaptive synthesis of cytochrome *c* has been suggested in the case of regenerating rat liver by Drabkin (1955). However, while the data support the view that cytochrome *c* is the limiting link in the pupal respiratory chain, they do not rule out the possibility that other factors, such as phosphate acceptors, may exert short-term effects on pupal respiration.

In conclusion, it is worth recalling that many animals other than diapausing pupae of the silkworm have a low respiration that is insensitive to carbon monoxide. Moreover, in many of these, such as diapausing eggs of grasshoppers and silkworms and unfertilized eggs of sea urchins, cytochrome oxidase is clearly present. The usual explanation for CO-insensitivity has been that respiration proceeded along tracks alternative to the cytochrome oxidase system (*cf.* Needham, 1942, p. 567). It is noteworthy, however, that in interpreting some of the very first experiments which showed this CO-insensitivity, Runnström (1930) suggested that cytochrome oxidase was not saturated with its substrate and this was the reason for CO-insensitivity in the sea urchin egg. In retrospect, it seems likely that this idea was sound and that the CO-insensitivity of the respiration of many systems is probably the result of an excess of cytochrome oxidase relative to some other component of the respiratory chain.

9. *Final theoretical considerations of carbon monoxide-insensitive respiration*

The basic premise underlying the arguments offered in the earlier sections of this discussion is that an excess of cytochrome oxidase can lead to a virtual carbon monoxide-insensitivity of a cytochrome oxidase-mediated respiratory chain. This is shown as follows. It is well known that carbon monoxide combines only with the reduced form of cytochrome oxidase (also called a_3):

$$(1) \qquad \mathrm{CO} + a_3^{++} \rightleftharpoons \mathrm{CO}\text{-}a_3^{++}; \qquad \mathrm{K} = \frac{(\mathrm{CO}\text{-}a_3^{++})}{(\mathrm{CO})(a_3^{++})}.$$

Equation (1) is the simple chemical equilibrium with a characteristic equilibrium constant that describes the interaction of reduced cytochrome oxidase (a_3^{++}) with carbon monoxide. This equation tells us that at a given concentration of carbon monoxide the amount of CO-a_3^{++} complex formed is determined solely by the *steady-state concentration* of reduced cytochrome oxidase.

Now the *steady-state concentration* of reduced (and oxidized) cytochrome oxidase is determined by the rate of electron transfer to cytochrome oxidase, and this, of course, is measured by the rate of oxygen consumption.

$$a_3^{++} + O_2 \rightarrow a_3^{+++} + O_2^- . \tag{2}$$

Equation (2) describes this *steady-state* between reduced and oxidized cytochrome oxidase. *It is important to note that equation (2) does not describe a simple chemical equilibrium but a steady-state* where the "apparent equilibrium constant" depends on the rate of electron transfer through the respiratory chain. Thus, if the rate of electron transfer to a_3^{+++} from the previous component in the chain is very slow, most of the cytochrome oxidase will be in the oxidized state and the ratio of a_3^{++} to a_3^{+++} will be small. Since the rate of electron transfer is measured by the rate of oxygen consumption, the "apparent equilibrium constant" for equation (2) will vary with the rate of oxygen consumption. This fact, incidentally, rules out the use of the usual Warburg formulation to describe quantitatively the effects of carbon monoxide on respiration, namely

$$\frac{N}{(1-N)}\frac{CO}{O_2} = K, \tag{3}$$

where "N" is the fraction of respiration not inhibited by carbon monoxide (Warburg, 1927). For, this formulation assumes that all the oxidase is in the reduced state, and hence that "the observed respiration is proportional to the amount of enzyme not combined with carbon monoxide" (Warburg, 1949, p. 78). Indeed, Warburg points out that in view of this assumption it is remarkable that there are cells for which his equation applies (p. 79).

When carbon monoxide is used as an inhibitor of cytochrome oxidase, the degree of inhibition of respiration depends upon the new steady-state reached by the system, in which both oxygen and carbon monoxide compete for reduced cytochrome oxidase. In this steady-state, some of the cytochrome oxidase is in the oxidized state, some is reduced and complexed with carbon monoxide, and the remainder is reduced and transferring electrons to molecular oxygen, *i.e.*, playing a role in respiration. The effect of carbon monoxide on respiration depends on the degree to which carbon monoxide decreases the concentration of reduced cytochrome oxidase that is transferring electrons to molecular oxygen. Since a_3^{++} must satisfy the equilibrium conditions of equation (1) and the steady-state conditions of equation (2), it becomes apparent that the amount of a_3^{+++} plays a major role in determining how much a_3^{++} remains to function in respiration. We thus see that the effect of carbon monoxide on respiration depends on the *fraction* of the total cytochrome oxidase in the reduced state. In other words, the effect of carbon monoxide on respiration depends upon the ratio of the actual rate of uninhibited respiration (as measured by the concentration of reduced cytochrome oxidase) to the maximum potential rate of respiration when virtually all the oxidase is kept in the reduced state (as measured by the total concentration of cytochrome oxidase). This ratio, $\frac{a_3^{++}}{a_3^{++} + a_3^{+++}}$, the fraction of the total oxidase in the reduced state, is what we ordinarily refer to as the "saturation" of cytochrome oxidase. When the saturation of cytochrome oxidase is high, the carbon monoxide sensitivity is high, and when the saturation is extremely low, the effect of carbon monoxide on respiration is insignificant. This can easily be seen when we consider two extreme cases, bearing in mind equations (1) and (2).

Let us examine a system in which the initial steady-state concentrations of a_3^{++} and a_3^{+++} are about equal (*i.e.*, a high saturation). In such a system, with a 20:1 CO/O_2 ratio an appreciable amount of CO-a_3^{++} can form. When the new steady-state is established in the presence of carbon monoxide, the ratio of the concentrations of a_3^{++} to a_3^{+++} is the same as before. However, the absolute concentration of both these components has been reduced considerably since a large part of the cytochrome oxidase is complexed with the carbon monoxide. As far as respiration is concerned, the significant reduction in a_3^{++} leads to a significant inhibition of respiration by carbon monoxide.

By contrast, consider a system in which the initial steady-state concentration of a_3^{+++} is much greater than the concentration of a_3^{++}. The presence of a CO/O_2 ratio of 20:1 will lead to the formation of only a small concentration of CO-a_3^{++} because of the low concentration of a_3^{++}. Indeed, when the difference between the concentrations of a_3^{+++} and a_3^{++} is very great (*i.e.*, a very low saturation), the total pool of cytochrome oxidase will not be significantly affected by carbon monoxide. As a result, the steady-state concentration of a_3^{++} will not be significantly diminished by the presence of carbon monoxide. Thus the CO-sensitivity of such a system is small.

From the above analysis we learn that an excess of cytochrome oxidase relative to other components of the respiratory chain will lead to CO-insensitivity of respiration. The same conclusion was reached independently by Harvey and Williams (1958b) using a different system and method of analysis.

One further theoretical consideration is crucial to the explanation offered above for CO-insensitivity. If the inhibition of cytochrome oxidase by carbon monoxide is a function of the *total* cytochrome oxidase present, then it must be possible for the transfer of electrons from the carrier part of the respiratory chain to proceed independently of a sterically specific arrangement of the chain components. In their review, Chance and Williams (1956) have discussed this possibility. They concluded that it was highly improbable that the chain components were fixed in position, and they presented two alternatives. Either the chain components were free to act by random collisions according to a modified law of mass action; or, they were fixed in such a manner that the prosthetic groups were free to rotate on an axis and be brought into apposition with adjacent chain components. In either case, electron transfer could proceed across chain components that were not immediately adjacent to one another. *Therefore, it seems possible for the carriers of the respiratory chain of the diapausing pupa to transfer electrons to a "pool" of cytochrome c oxidase. This pool of cytochrome oxidase can manage all of the oxidations, even in the presence of inhibitors, as long as there is sufficient uninhibited enzyme present to meet the needs of electron transfer.* In short, it appears possible for an excess of cytochrome oxidase in tissues to account for the CO- and azide-insensitivity of respiration and of various physiological functions such as heart-beat.

The arguments presented in the previous sections persuade us that this is the situation in most of the tissues of diapausing silkworm pupae.

The experiments reported in Section 1A were performed in collaboration with Dr. Roger D. Smith. We gratefully acknowledge the helpful criticisms of Dr. David P. Hackett, Dr. Conrad S. Yocum, Dr. Carroll M. Williams, and Dr. Howard M. Lenhoff.

SUMMARY

1. To characterize the respiratory enzyme chain that functions during diapause, the respiration of diapausing pupae of the Cecropia, Cynthia, Promethea and Polyphemus silkworms was measured in the presence of specific mixtures of oxygen, nitrogen and carbon monoxide, after injection of various metabolic inhibitors and after injury.

2. Pupal respiration is at best only slightly inhibited by carbon monoxide and is often stimulated. Whatever CO-sensitivity there is occurs only in pupae with high basal metabolic rates. Moreover, when respiration is accelerated by injecting dinitrophenol (DNP), or by injury, this evokes an enhanced sensitivity to carbon monoxide. Indeed, it appears that the fraction of respiration sensitive to carbon monoxide is a function of the rate of oxygen consumption of the silkworm at all stages.

3. Reducing external oxygen tension to 2% fails to inhibit oxygen consumption, but increases markedly the CO-sensitivity of pupal respiration. Thus low oxygen tensions seem to unmask CO-sensitivity.

4. Pupal respiration is insensitive to azide concentrations as high as 5×10^{-4} *M*. However, the azide-sensitivity, like the CO-sensitivity, increases markedly when pupal respiration is stimulated by DNP or injury.

5. Antimycin A at a concentration of 10^{-6} *M* inhibits less than one-third of normal pupal respiration or DNP-stimulated respiration. Compared to other organisms diapausing pupae are resistant to this inhibitor of the cytochrome *c* reductase system.

6. Dinitrophenol at a concentration of 5×10^{-4} *M* stimulates pupal respiration an average of 12-fold and as much as 16-fold. These are among the largest DNP-stimulations ever recorded. Although pupae with high basal metabolic rates are less stimulated proportionately by DNP than are pupae with low basal metabolic rates, they develop a greater over-all respiration under the influence of DNP.

7. Dinitrophenol-stimulated respiration is inhibited by carbon monoxide. The higher the DNP-stimulated respiration, the greater the inhibition by carbon monoxide. From this and other evidence it appears very likely that DNP accelerates the turnover of one or several components of the respiratory chain while having a lesser effect on cytochrome oxidase.

8. Dinitrophenol delays the appearance of injury-stimulated respiration, suggesting that the development of this increased respiration requires phosphate bond energy. Furthermore, exposure to carbon monoxide causes the death of injured pupae indicating that injury respiration is obligatory and involves the synthesis of new respiratory components.

9. Newly molted pupae not yet firmly in diapause do not respond to wounding with an injury respiration and their respiration is sensitive to carbon monoxide. These findings are correlated with their high respiratory rate.

10. The modes of action of the several inhibitors within diapausing, injured, and developing insects are considered in detail and a new explanation is proposed to account for the CO-, azide-, and cyanide-insensitivity of pupal respiration.

11. It is concluded that the insensitivity of diapausing pupae to inhibitors of cytochrome oxidase results from an excess of this enzyme over its functional requirements in the pupal respiratory chain. This concept is examined in detail and found to be theoretically sound. Evidence is presented that the limiting link in the respiratory chain is cytochrome *c*. Thus, contrary to earlier conceptions, it appears that cytochrome oxidase is the principal terminal oxidase during diapause as well as during all the other stages of the life history, and that the CO-insensitivity of pupal respiration stems from a great excess of cytochrome oxidase relative to cytochrome *c*.

12. The increased CO- and azide-sensitivity of pupal respiration after injection of DNP or injury results from an increase in the saturation of cytochrome oxidase provoked on the one hand by an increase in the turnover rate of cytochrome *c*, and on the other by the synthesis of cytochrome *c*.

13. It is suggested that the CO-insensitivity of the respiration of other organisms may be the result of an excess of cytochrome oxidase relative to some other components of the respiratory chain.

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PERIODICITY OF MITOSIS AND CELL DIVISION IN THE EUGLENINEAE[1]

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In the course of an investigation into the division cytology of flagellates of the class Euglenineae, it became necessary to determine the time and rate of mitosis for each of the forty species under examination. The present paper deals with the periodicity of mitosis revealed in the twenty species studied in detail for this feature, and relationship of the periodicity to the day-night cycle. An account of the structure and division of the cell and nucleus will be published separately (see Leedale, 1958a, 1958b).

MATERIAL AND METHODS

1. *Species studied*[2]

The three main sources of material have been my own wild collections, the Cambridge Culture Collection of Algae and Protozoa, and the Sammlung von Algenkulturen, Göttingen. All species have been isolated by Professor E. G. Pringsheim or myself, with the exception of *Trachelomonas grandis* which was isolated by Singh (Singh, 1956) and sent to me by Professor H. C. Bold.

The names of species are corrected according to Pringsheim (1956) for the genus *Euglena*, and to Huber-Pestalozzi (1955) for the remaining genera. Colorless species are indicated by an asterisk.

* *Astasia klebsii* Lemmermann
Colacium mucronatum Bourrelly
Cryptoglena pigra Ehrenberg
* *Distigma proteus* Ehrenberg em. Pringsheim
Euglena acus Ehrenberg
Euglena deses Ehrenberg
Euglena gracilis Klebs (strain "T," green form)
* *Euglena gracilis* Klebs (strain "T," colorless form)
Euglena gracilis Klebs (strain "Z," green)
Euglena spirogyra Ehrenberg
Euglena viridis Ehrenberg
Eutreptia pertyi Pringsheim
Eutreptia viridis Perty

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[1] From a study carried out in the Botany Departments of Queen Mary College, London, and The Durham Colleges; some of the results were included in a thesis approved for the degree of Doctor of Philosophy in the University of London. My thanks are due to Dr. M. B. E. Godward of Queen Mary College and Professor E. G. Pringsheim of the University of Göttingen for their help and advice.

[2] I would like to thank Professor E. G. Pringsheim, Mr. E. A. George of Cambridge and Professor H. C. Bold for supplying me with material.

* *Hyalophacus ocellatus* Pringsheim
Lepocinclis ovum var. *buetschlii* (Conrad) Huber-Pestalozzi
Lepocinclis steinii Lemmermann em. Conrad
* *Menoidium cultellus* Pringsheim
* *Peranema trichophorum* (Ehrenberg) Stein
Phacus pusillus Lemmermann
Phacus pyrum (Ehrenberg) Stein
Trachelomonas bulla Stein em. Deflandre
Trachelomonas grandis Singh

2. *Cultivation*

Cells were isolated from wild collections by the micropipette method (Pringsheim, 1946a). All species except *Peranema trichophorum* were grown in soil-water tubes (biphasic culture, Pringsheim, 1946a, 1946b) with a wheat grain, starch or ammonium magnesium phosphate beneath the soil. *Eutreptia* spp. were grown in tubes with 50% sea-water. *Peranema trichophorum* was grown in soil extract containing 0.5% milk.

In addition to the biphasic cultures, green and colorless forms of *Euglena gracilis* (strain "T") were cultivated in 0.2% Difco beef extract, or "SATBY" (0.1% sodium acetate, 0.2% Difco tryptone, 0.1% Difco beef extract, 0.2% Difco yeast extract, in distilled water).

Cultures were hung in a north-facing window or in temperature-controlled cabinets with either incandescent or fluorescent lighting on a time-switch. The cultures were grown at a standard temperature of 20° C.

General Features of the Cultures

A biphasic culture of any species of the Euglenineae has a typical growth pattern. Sub-culturing to a new tube with a heavy inoculum is followed by a lag-period of two to three days during which time there are few or no divisions. This is followed by a period of multiplication which is eventually slowed and halted by overcrowding of the medium. There is an upper limit of number of cells per ml. of medium (the "culture saturation point") at which cell multiplication falls to a low rate. This effect is not caused by exhaustion of the medium; if the cells of a "saturated" culture are centrifuged off and the medium re-inoculated, the culture builds up as quickly as before, and this can be repeated several times.

According to the size of the inoculum, the division rate, and the "culture saturation point" of the species concerned, the increase in cell numbers may continue for one to twelve months. It is the mitotic rhythms occurring during this period of multiplication which are the subject of this paper.

Mitotic Periodicity in Green Species

Fixations made at two-hourly intervals for several (not successive) 24-hour periods showed that all green species of the Euglenineae had mitosis confined to the dark period when growing in biphasic culture under natural light conditions.

The restriction of nuclear division to the dark period was examined in detail in

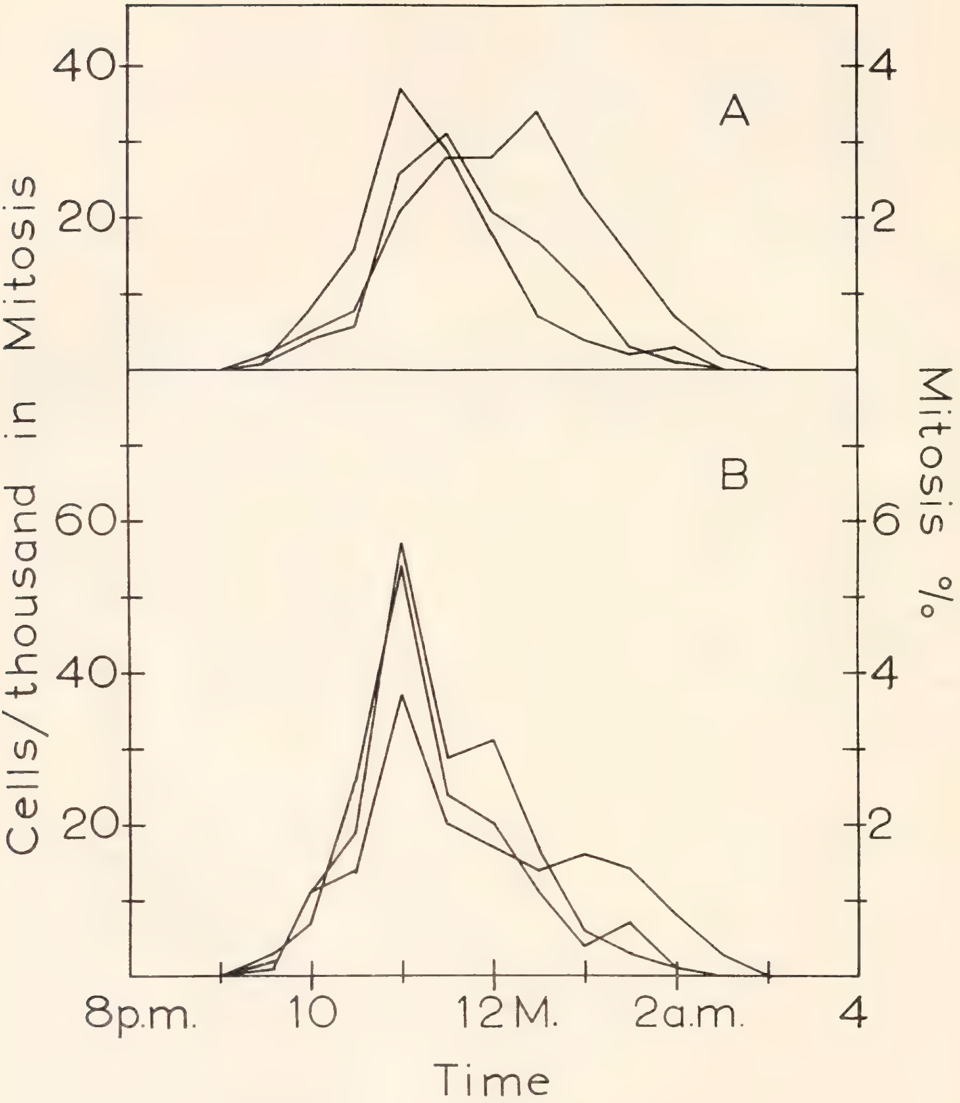
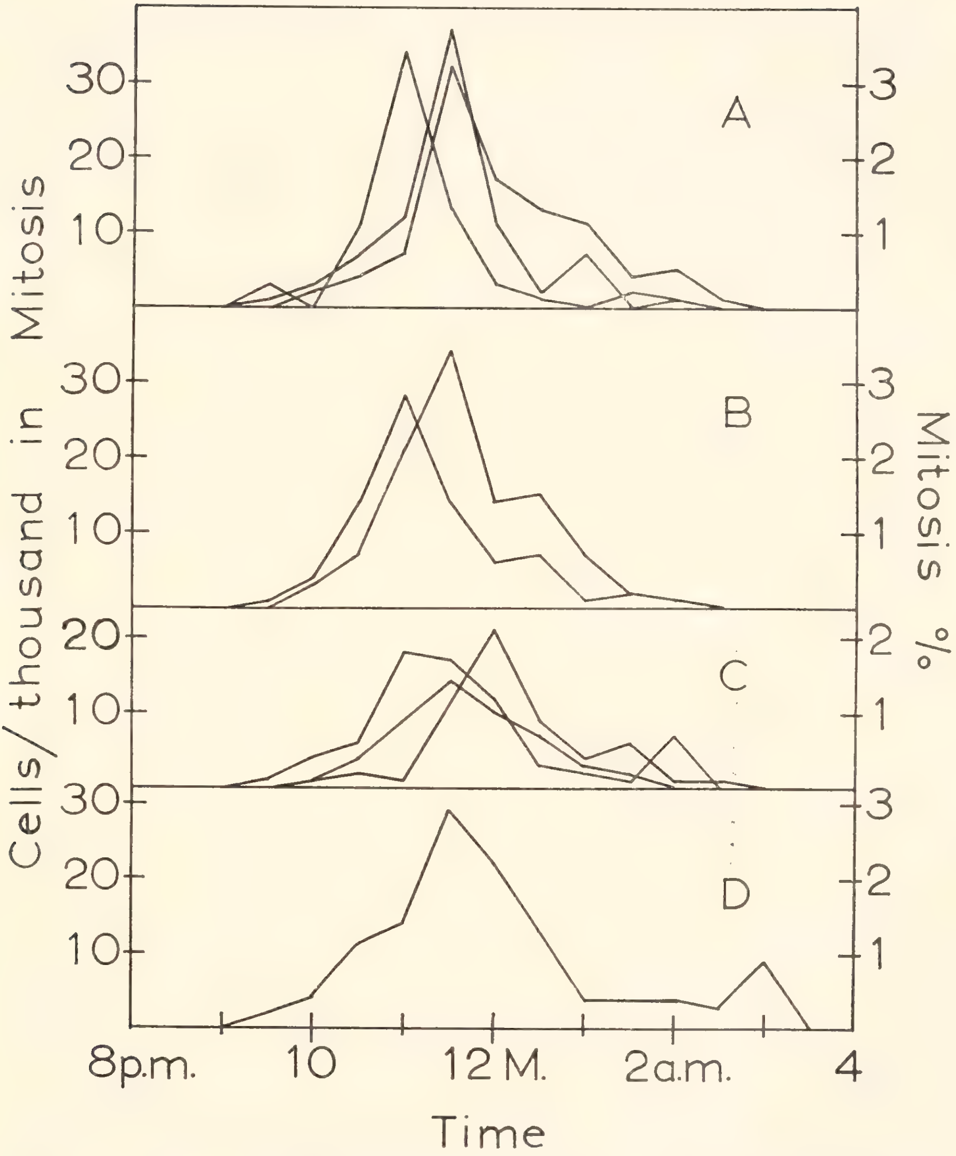


FIGURE 1.

fifteen green species. Fixations were made at half-hourly intervals from the onset of darkness on one, two or three consecutive nights, the material for any one series being taken from the same culture tube. Five hundred cells were counted in each of two preparations from each fixation and the number of cells in mitosis and cell division noted. The results of these counts were similar for all species and are recorded graphically for six representative species in Figures 1 and 2.

Mitosis began one to two hours after the onset of darkness. In *Euglena spirogyra* (Fig. 1, A), *Euglena viridis* (Fig. 1, B) and *Eutreptia pertyi,* mitosis began at the same time on each of three successive nights. The mitotic maximum



FIGURES 1 and 2. The number of cells per thousand in mitosis at half-hourly intervals during one, two or three consecutive nights, plotted as mitosis percentage against time. All results are for biphasic cultures growing in the natural day-night cycle, the dark period beginning at 8 P.M. FIGURE 1. Green species: A, *Euglena spirogyra;* B, *Euglena viridis.* FIGURE 2. Green species: A, *Phacus pusillus;* B, *Phacus pyrum;* C, *Trachelomonas bulla;* D, *Trachelomonas grandis.*

occurred from 2½ to 4½ hours after the onset of darkness in all species. The maxima for *Euglena viridis* (Fig. 1, B), *Eutreptia pertyi* and *Phacus pusillus* (Fig. 2, A) occurred at the same time on three successive nights; those for *Euglena spirogyra* (Fig. 1, A) covered a two-hour period within three nights. The span of the nightly period during which mitosis occurred ranged from three to six hours in the different species. The mean maximum percentage of cells undergoing mitosis each night is recorded in Table I.

Recording the number of cells at each stage of mitosis in each fixation produced a more detailed picture of the periodicity. The results for *Euglena spirogyra* for one dark period (Fig. 3) illustrate the complete restriction of nuclear and cell division to within a five-hour period, beginning approximately two hours after the onset of darkness. Successive maxima of the mitotic stages occur, a wave of prophases being followed by waves of metaphases, anaphases, telophases and cell cleavage. This pattern was repeated in other cultures of the same species and by other species, the relative size and span of the maxima varying according to the duration of the stages of mitosis in the different species.

TABLE I

The mean maximum percentage of cells undergoing mitosis each night in green species of the Euglenineae in biphasic culture at 20° C.

Species	Mean maximum %
Colacium mucronatum	2.6
Cryptoglena pigra	1.8
Euglena acus	1.9
Euglena deses	2.2
Euglena gracilis "T"	4.2
Euglena gracilis "Z"	5.7
Euglena spirogyra	3.4
Euglena viridis	4.9
Eutreptia pertyi	3.5
Eutreptia viridis	2.3
Lepocinclis ovum var. *buetschlii*	6.8
Lepocinclis steinii	1.3
Phacus pusillus	3.4
Phacus pyrum	3.1
Trachelomonas bulla	1.8
Trachelomonas grandis	2.9

Further series of fixations over a period of one year showed that no matter at what time of the clock the natural dark period began, mitosis began one to two hours later, the percentage of cells dividing each night being of the same order for any one species (at 20° C.). There was no variation in the mitotic rate in relation to day-length.

Examination of the same culture over a period of several months showed the multiplication period to be discontinuous. Weeks with divisions occurring every night were interspersed with occasional days when no divisions occurred.

The introduction of an artificial dark period during the natural light period affected mitotic periodicity in all the green species. If the artificial dark period was begun three hours or less before the natural one, mitosis occurred, but in a lower percentage of cells than usual. When the artificial dark period was introduced six hours or more before the natural one was due to begin, divisions rarely occurred. The shortest day-length after which mitosis would occur was approximately twelve hours.

No mitosis or cell division could be induced in any green species in biphasic culture in any conditions or intensity of artificial lighting, either direct or diffused, incandescent or fluorescent. Attempts to reverse the mitotic periodicity in a temperature-controlled cabinet with lighting on a time-switch were unsuccessful, the cells becoming quiescent with no divisions occurring. Similarly, no mitosis occurred in either continuous light or continuous darkness. Returned to natural light conditions after such treatment, the cells recovered their full division rate within a day if the treatment had been short, but less quickly if the treatment was prolonged.

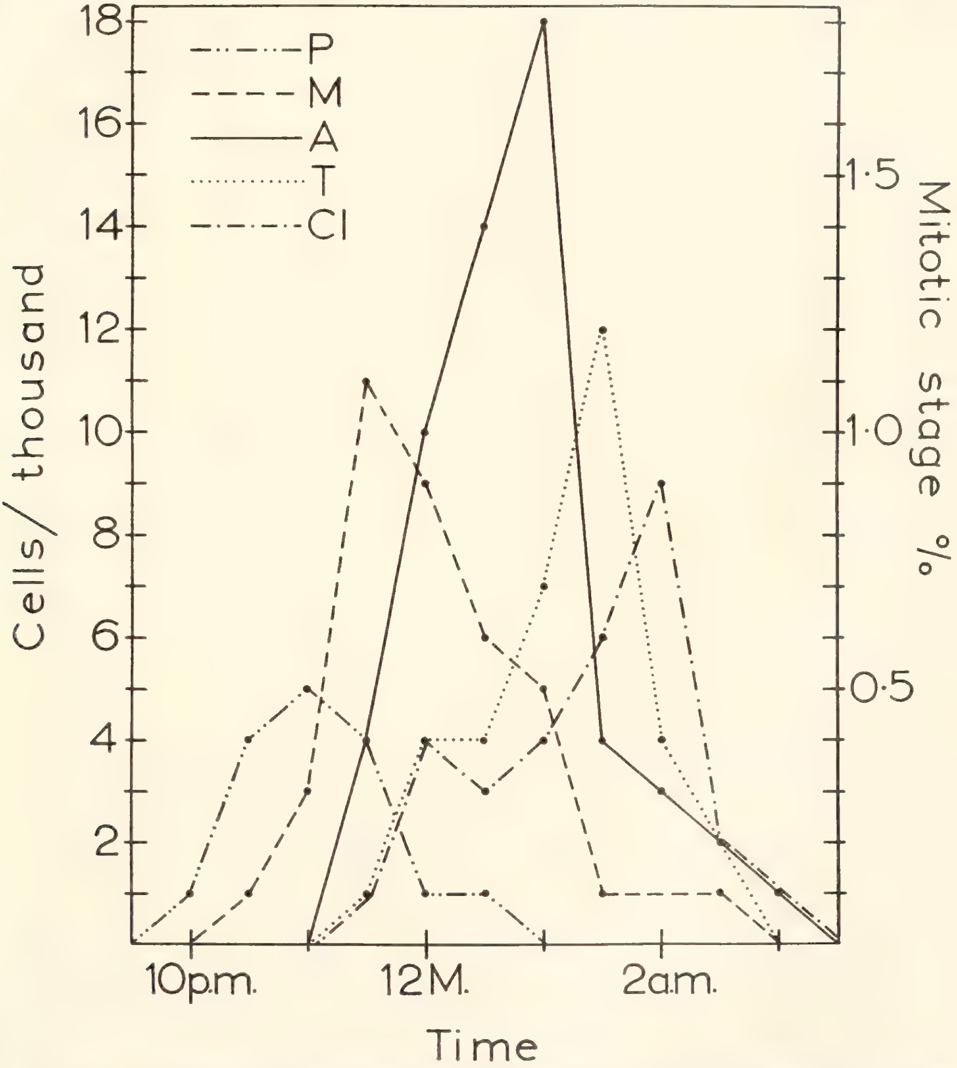


FIGURE 3. Mitosis in *Euglena spirogyra*. The number of cells per thousand in prophase (P), metaphase (M), anaphase (A), telophase (T) and cell cleavage (Cl) at half-hourly intervals during one night, plotted as percentage of each mitotic stage against time. The results are for a biphasic culture growing in the natural day-night cycle, the dark period beginning at 8 PM.

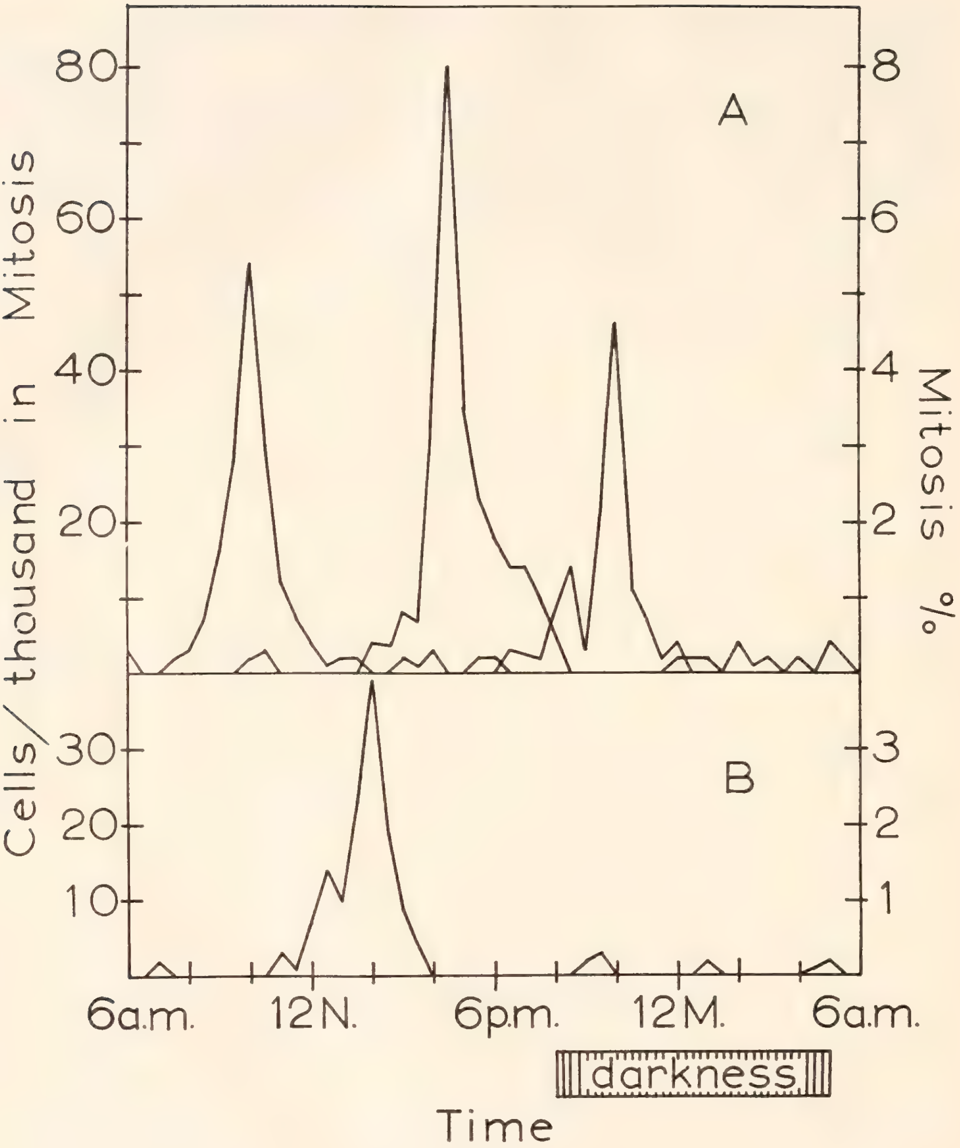
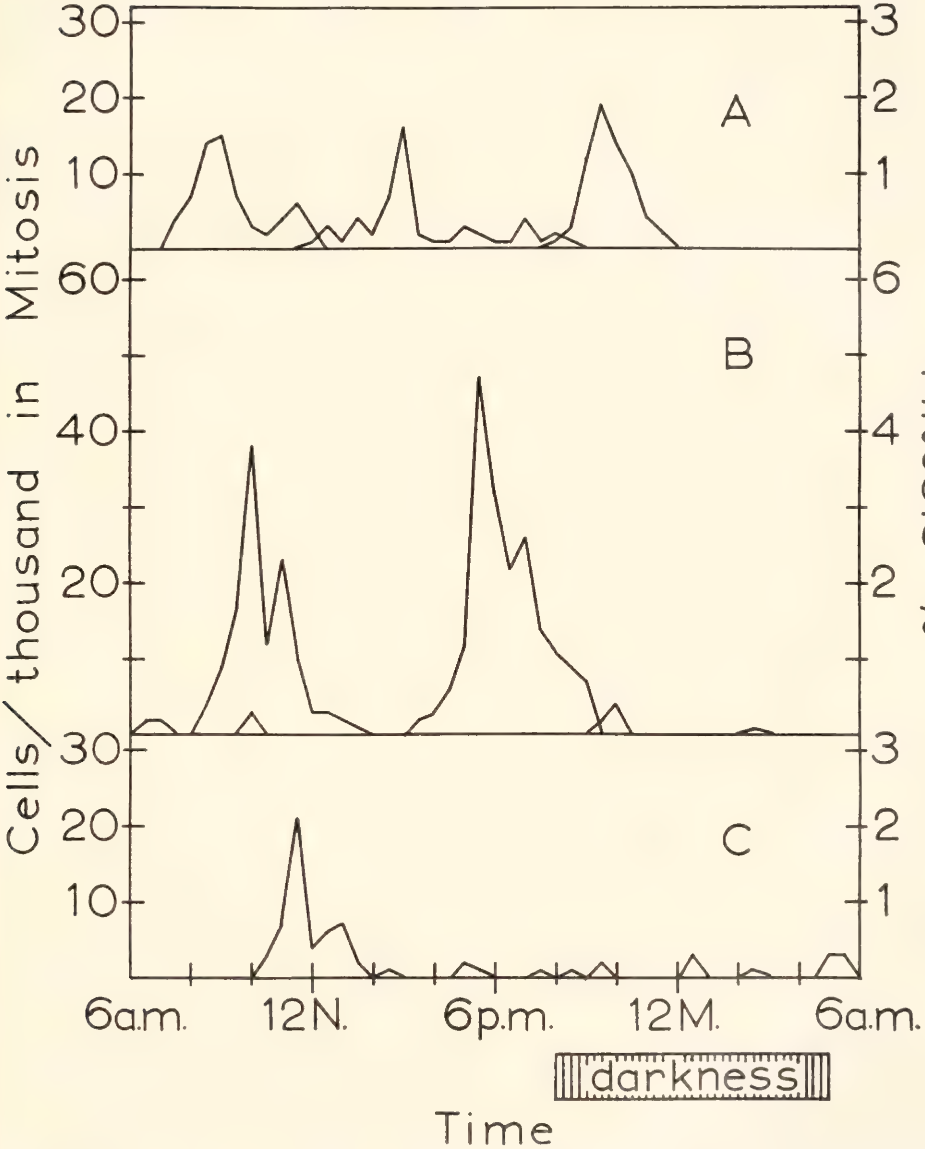


FIGURE 4.

Once mitosis had begun, it proceeded to conclusion even if the dividing cell was then subjected to light. However, if light was introduced less than an hour after the onset of darkness, no mitosis occurred. If a dark period of more than one hour followed a full-length day and artificial light was then introduced, some cells underwent a complete mitotic division, though on first examination *no cells could be found in mitosis, not even in prophase.*

Euglena gracilis was the only species in which the time and rate of mitosis in biphasic culture could be compared with those in a rich liquid medium. The



FIGURES 4 and 5. The number of cells per thousand in mitosis at half-hourly intervals during one, two or three (not consecutive) 24-hour periods, plotted as mitosis percentage against time. All results are for biphasic or milk cultures growing in the natural day-night cycle. FIGURE 4. Colorless species: A, *Astasia klebsii;* B, *Distigma proteus.* FIGURE 5. Colorless species: A, *Hyalophacus ocellatus;* B, *Menoidium cultellus;* C, *Peranema trichophorum.*

mitotic rhythm shown by the green form of strain "T" in biphasic culture was absent in 0.2% beef extract or "SATBY" medium. During the period of rapid multiplication prior to crowding of the culture, a fixation at any time of day or night showed from 5–6% (beef extract) or 8–10% ("SATBY") of the cells undergoing mitosis (at 20° C.). At 30° C. the mitotic rate of *Euglena gracilis* "T" in "SATBY" was 25–30%. In biphasic culture, maximum division rates were obtained at 20° C.; raising or lowering the temperature by five degrees resulted in a fall in division rate.

MITOTIC PERIODICITY IN COLORLESS SPECIES

Fixations made at half-hourly intervals over 24-hour periods showed that a constant rate of mitosis was not maintained in any colorless species of the Euglenineae in biphasic culture, bursts of mitotic activity alternating with periods when mitosis was almost completely absent.

The results for 24-hour series of half-hourly fixations are recorded for the five species studied in Figures 4 and 5. In addition to these series where a division maximum occurred at some time during the 24-hour period, numerous series contained no divisions or a few divisions scattered throughout the period. Many single fixations at different times of day or night contained cells in mitosis.

TABLE II

The maximum percentage of cells recorded in mitosis at any one time in colorless species of the Euglenineae in biphasic or milk culture at 20° C.

Species	Maximum %
Astasia klebsii	8.0
Distigma proteus	3.9
Hyalophacus ocellatus	1.9
Menoidium cultellus	4.7
Peranema trichophorum (in milk)	2.1

Mitotic maxima occurred at any time of the clock. In none of the five species did the periods of major mitotic activity bear any relationship to the alternating light and darkness of the natural day-night cycle. The recorded maxima for *Astasia klebsii* (Fig. 4, A) occurred at 10 AM, 4:30 PM and 10 PM; those for *Hyalophacus ocellatus* (Fig. 5, A) at 9 AM, 3 PM and 9:30 PM. The time-spans of the major periods of mitotic activity ranged from 3½ to 8½ hours.

The highest percentage of cells obtained dividing at any one time is recorded for each species in Table II. The percentages of cells dividing at different times on different dates were of the same order for some species (Fig. 5, A and B) but not for *Astasia klebsii* (Fig. 4, A).

The irregularly spaced bursts of major mitotic activity in the colorless species continued in alternating artificial light and darkness, in continuous light, and in continuous darkness.

The colorless form of *Euglena gracilis* "T" growing in 0.2% beef extract or "SATBY" medium behaved as did the green form in these media, exhibiting no periodicity of mitosis, regular or irregular. A continuous division rate of 6–7% was maintained in "SATBY" at 20° C., the rate increasing to 30–35% at 30° C.

DISCUSSION

Mitotic rhythms have been recorded for higher plants by Lewis (1901), Kellicott (1904), Karsten (1915), Laughlin (1919), Stalfelt (1919), Friesner (1920), Tischler (1921), Abele (1925), Brown (1951) and Jensen and Kavaljian (1958).

The rhythm, in most cases thought to be endogenous, has been related to the onset of germination, the balance between cell elongation and division, or light periodicity. Lewis (1901) and Karsten (1915) found that the times of the maxima altered when light conditions were changed, but Friesner (1920) found the maxima were independent of light changes. Stalfelt (1919) and Brown (1951) state that the mitotic rhythm of higher plants is exogenously imposed by the day-night cycle, disappearing when the plants are grown in continuous darkness. No evidence of mitotic rhythms in higher plants was found by Winter (1929) or Gray and Scholes (1951).

Mitotic rhythms in animals have been recorded by Ortiz-Picon (1933), Carleton, (1934), Cooper and Schiff (1938), Cooper and Franklin (1940), Blumenfeld (1942, 1943), Bullough (1948) and Milletti (1950). The rhythm has been related to the activity cycle, a higher division rate occurring when the animal is at rest (Cooper and Schiff, 1938; Bullough, 1948; Milletti, 1950). Kalmus (1935) has recorded an exogenous rhythm of cell division for *Paramecium*.

Twenty-four-hour rhythms of mitosis have been recorded for a number of algae. Division occurring exclusively at night has been recorded for species of the genera *Cladophora* and *Stigeoclonium* (Braun, 1851), *Spirogyra* (Braun, 1851; Famintzin, 1867; Sachs, 1874; Strasburger, 1880), *Zygnema* (Kurssanow, 1912), and *Vaucheria, Hydrodictyon* and *Ulothrix* (Sachs, 1874), whilst Karsten (1918) found three maxima in each 24-hour period for species of *Closterium, Cosmarium* and *Mesotaenium*. Wildeman (1891) found no mitotic rhythm in *Spirogyra*. The present author has found mitosis almost entirely confined to the dark period in species of *Hydrodictyon, Ulothrix, Mougeotia, Spirogyra, Zygnema, Closterium, Cosmarium* and *Staurastrum* in biphasic culture. The rhythm was exogenous in these species, and mitosis could be produced at any time of the clock by adjusting the time of the dark period in a culture cabinet. Some species of *Spirogyra* and *Zygnema* underwent mitosis in continuous light.

A nocturnal periodicity of mitosis in euglenoid species has been mentioned by Dangeard (1902) for species of *Euglena, Phacus* and *Trachelomonas,* Baker (1926) for *Euglena gracilis* in a split pea infusion, Ratcliffe (1927) for *Euglena spirogyra* in modified Doflein's medium, S. R. Hall (1931) for the parasitic *Euglena leucops* Hall when in its host, a species of *Stenostomum,* Gojdics (1934) for *Euglena deses* in 0.1% beef extract, Johnson (1934) for *Colacium vesiculosum* Ehrbg. and Chu (1946) for *Euglena* spp. in biphasic culture. Only sparse growth was possible in several of the media recommended by these authors. Lackey (1929) has made the only record of a division maximum at night in a colorless species (*Entosiphon sulcatum* (Duj.) Stein, grown in a cracked wheat medium) and suggests it might be explained on phylogenetic grounds. This is to be doubted since the nocturnal rhythm of the green species is not endogenous and no such rhythm is present in the five colorless species investigated in the present study. Lackey records some divisions during the day and it is probable that his division maxima occurred during the dark period by chance, without being related to it.

The mitotic maxima recorded for higher plants and animals are *increases* over a continuous low division rate. As would be expected, in organisms composed of many cells arranged in tissues, some of which are specifically concerned with cell division, mitosis occurs throughout the 24-hour period in the division sites. Diurnal rhythms, whether in areas directly affected by light or not (root-tip meristems in plants, bone marrow in animals), can be related to metabolic rhythms, maximum mitosis occurring during the period of minimum activity.

In unicellular organisms the division of labor between cell growth and mitosis is often in time rather than in space. A cell tends to divide during a period of minimum activity *of that particular cell.* Thus green unicells and filamentous green algae often have a rhythm of mitosis which is closely related to the rhythm of photosynthetic activity in the day-night cycle.

Such a relationship is exhibited by the Euglenineae. Green species, when living autotrophically, divide only in the dark, and an almost full period of natural daylight is necessary before mitosis will occur in the ensuing dark period. A threshold period of darkness is required for the induction of mitosis, but once induction has occurred, the mitotic process will begin and proceed to completion, even though the cell be subjected to light before its nucleus has begun the anterior migration which is the first sign of approaching mitosis. This induction precedes prophase by a period of up to one hour, since the threshold period for induction is approximately one hour after the onset of darkness, whilst the first prophases in all species appear one to two hours after darkness. The final inductions would then be occurring approximately three hours later, since the last prophases appear four to five hours after darkness (Fig. 3).

It has been shown in *Euglena gracilis* that the nocturnal periodicity of mitosis is removed by the stimulus of a rich food supply, the heterotrophic ("chemotrophic") mode of nutrition of this species in beef extract or "SATBY" being unrelated to the day-night cycle.

Mitosis in colorless species of the Euglenineae in biphasic culture shows an irregular periodicity which is not related to the natural day-night cycle. The heterotrophic mode of nutrition of the colorless species is also independent of light.

The factor deciding which cells in a culture divide during any one period of mitosis is probably cell age (reflecting cell size and cell maturity) in both green and colorless species. If 5% of the cells of a biphasic culture of a green species divide each night in turn, the span of a generation will be 20 days. In *Euglena gracilis* in "SATBY" at 30° C., with a division rate of 25–35%, the generation span cannot be more than 8–12 hours.

The nocturnal rhythm of mitosis is presumably present in green species in the wild when the supply of nutrients is low. An influx of rich organic nutrients will remove the periodicity and, when combined with optimum temperature and pH, may result in the sudden euglenoid "blooms" which often occur in bog-pools, farmyards, ponds and lakes.

SUMMARY

1. The periodicity of mitosis and cell division has been investigated in 15 green and 5 colorless species of the Euglenineae.

2. Green species in biphasic culture under natural light conditions have mitosis confined to the dark period. Mitosis begins one to two hours after the onset of darkness, each species having a predictable percentage of cells dividing each night. There is a threshold period at the beginning of the dark period after which mitosis cannot be inhibited by light. The mitotic rhythm is exogenous, being removed by growth in artificial light or darkness (resulting in no mitosis), or in a rich organic medium (resulting in continuous mitosis at a constant rate).

3. Colorless species in biphasic culture under any light conditions have an irregular mitotic periodicity, bursts of mitosis occurring at any time of the clock and alternating with periods in which mitosis is almost absent.

4. It is suggested that the presence or absence of regular or irregular mitotic periodicity is related to the different modes and rates of nutrition of green and colorless species in various conditions of light and darkness, and in various media.

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THE HORMONAL CONTROL OF METABOLISM IN CRUSTACEANS. IX. CARBOHYDRATE METABOLISM IN THE TRANSITION FROM INTERMOULT TO PREMOULT IN CARCINIDES MAENAS

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Recent reviews of the metabolic events in the intermoult cycle of decapod crustaceans, and of the hormonal control of these events, have emphasized the fragmentary nature of our present knowledge (Knowles and Carlisle, 1956; Scheer, 1957). Particular interest centers around the metabolism of carbohydrate, which is known from the work of Renaud (1949) to undergo considerable changes in the course of the intermoult cycle. The present report is based on a study of a laboratory population of approximately 100 specimens of the crab *Carcinides* (=*Carcinus*) *maenas,* in which the content of total carbohydrate, total soluble polysaccharide, blood carbohydrate, blood lipochromes, and total non-protein nitrogen was determined on the individuals in samples drawn at intervals from the population.

MATERIALS AND METHODS

The animals were taken from a lagoon north of Banyuls-sur-Mer and brought into the laboratory on October 11; they were maintained throughout the experiment in large aquaria in running sea water, and fed regularly on mussels. Examination of the animals showed them all to be in the hard-shelled condition (stages C_2 through D_2 of Drach, 1939), but closer determination of intermoult cycle stage was not made until the animals were killed for analysis. Most of the animals were males, and only males were used for the studies reported, to avoid complications arising out of sexual differences. On October 14, November 13, and December 1, samples of 20 to 30 crabs, selected at random, were drawn from the group, and the eyestalks were removed from every second animal. Mortality was very low. It is probable that a few animals moulted during the period of the experiment, but cannibalism prevented any certain determination of this.

Eight to ten days after the sampling, the animals were extracted for analysis. The stage in the intermoult cycle was carefully determined, using the criteria of Drach (1939). For this study, an exact determination of the division between the end of the intermoult period (C_4) and the beginning of the premoult period (D_1) was essential. Accordingly, microscopic examination of the external branchial epipodite of the first maxilliped was made to determine the presence of newly formed setae beneath the old integument of this appendage. The presence of even the most rudimentary new setae was taken as an index of the beginning of stage D_1. These rudimentary setae can be detected only by careful microscopic examination under good illumination by transmitted light.

A blood sample was taken by bleeding from a cut walking leg. The animal was then quickly cut up into 50–75 ml. of 5% trichloracetic acid; Renaud (1949) had already shown that the elaborate precautions to prevent glycolysis which are necessary in mammals are not as important in crabs. The mixture of acid and tissue was transferred to an electric blender (Cadillac Atomixer), and blended at 8000 rpm. for 3 minutes. The mixture was rapidly filtered with suction, and the residue returned to the blender with a second portion of acid for a second extraction. The blender and residue were washed with a third portion of acid. The combined filtrates were then diluted in a volumetric flask, usually to 250 ml., and stored in the refrigerator until analyzed, always within a few days.

Blood carbohydrate was determined on some samples by the anthrone method of Roe (1955). One ml. of blood was collected by dripping from a cut walking leg, into a calibrated tube. One ml. of 5% trichloracetic acid was added with mixing, and the mixture was centrifuged. One ml. of the supernatant was then transferred to a second tube for colorimetric determination. Blood lipochromes were determined on other samples. To 1 ml. of blood, 5 ml. of acetone were added. The acetone solution was then extracted with 2 ml. of petroleum ether, the ether layer was washed with water, dried with solid KOH, and diluted to 5 ml. with petroleum ether. The concentration of lipochromes was then read in the spectrophotometer at 450 $m\mu$ against a petroleum ether blank. The measurements are given as optical densities, since the exact nature of the lipochromes involved is not known.

Total carbohydrate was determined by the anthrone method (Roe, 1955). One hundred microliters (μl) of the extract were transferred to a tube with a micropipette, and diluted to one ml. for colorimetric determination. Polysaccharide was determined by the same method. To 1 ml. of extract, 5 ml. of 95% ethyl alcohol were added, and the mixture allowed to stand overnight in the refrigerator. The tubes were centrifuged, the precipitate carefully drained, and suspended in 10 ml. of distilled water. A one-ml. sample of this suspension was used for colorimetric analysis. The anthrone method has the advantage for this study that it determines a variety of carbohydrates, and relatively few other naturally occurring compounds. All results are expressed in terms of glucose equivalents.

Non-protein nitrogen (NPN) was determined on 10-ml. samples of the extract, using the micro-Kjeldahl digestion method of Hiller *et al.* (1948) and distilling the digested mixture into 0.1 N HCl in an all-glass still. Ammonia nitrogen (NH_3N) was separated by distilling the undigested extract in the same still. The final determination of ammonia in both cases was colorimetric, using the Nessler reagent.

RESULTS

In the first sample, examined 11 to 19 days after collection, 10 out of 16 animals (63%) were in the C_4 stage (late intermoult) of the intermoult cycle; the remainder were in the D_1 stage (early premoult). In the second sample, examined 43 to 45 days after collection, the proportion of C_4 animals was 52% (12 C_4, 8 D_1, 3 D_2). In the third sample, the proportion was 32% (7 C_4, 15 D_1). These values suggest that the population from which the samples were drawn was undergoing a steady progression towards the moult. The χ^2 test shows that the proportion of C_4 animals in the third sample is significantly less than in the first, at the 5% level of probability.

In the first sample, only one of the 10 C_4 animals had blood clearly pigmented with lipochromes, while 5 of the 16 D_1 animals had blood so pigmented; no quantitative determinations were made in this series. In the second sample, 5 of the 12 C_4 animals and 6 of the 11 D_1 animals had lipochromes clearly evident in the blood; quantitative measurements were made on these 11 animals, and are presented in Table I. For the third sample, quantitative measurements were made on all the animals, and are presented in Table I. From the results on the third sample, in which traces of lipochrome are found in nearly all specimens, it appears that the level for qualitative detection of lipochromes lies at about 0.05 on the density scale used to express concentrations. On this basis, we would conclude that only one of the 7 C_4 animals of the third sample had substantial amounts of lipochrome in the blood, while 7 of the 15 D_1 animals had such amounts. If we apply the χ^2 test to these values, we find that the frequency of occurrence of easily observable amounts of lipochrome in the blood is not significantly different from 1 in 10 animals for the C_4 stage in samples 1 and 3, but is significantly different, at the 10% level of probability or better, for all the other groups. The 1:10 ratio observed in C_4, sample 1, is also significantly different from the 1:2 ratio observed in D_1, sample 3.

TABLE I

Lipochromes in the blood of Carcinides maenas. Optical density at 450 mμ of a petroleum ether extract, volume 5 ml., from 1 ml. of blood. The values for sample 2 (see text) represent only animals in which blood lipochromes were qualitatively evident

	C_4				D_1			
Stage condition	Normal		Eyestalkless		Normal		Eyestalkless	
	Animal	Density	Animal	Density	Animal	Density	Animal	Density
Sample 2	22	0.072	24	0.064	31	0.150	35	0.070
	36	0.065	29	0.059	34	0.077	23*	0.157
			33	0.112	40	0.088		
					42*	0.076		
Sample 3	45	0.063	44	0.016	47	0.000	48	0.000
	49	0.010	46	0.020	55	0.035	50	0.055
	51	0.015	54	0.010	57	0.030	52	0.020
	53	0.012			59	0.026	56	0.050
					61	0.105	58	0.090
					63	0.095	60	0.010
					65	0.066	62	0.210

* Stage D_2.

The mobilization of lipochromes from the digestive gland to the integumentary tissues is an important part of the preparation for the moult, and all of the D_1 animals in this study showed deposits of pigment in the region of the membranous layer of the integument; indeed, this characteristic appears to be a fairly reliable means of detecting the beginning of the premoult period. The appearance of lipochrome in substantial amounts in the blood may therefore be taken as an indication of the beginning of preparations for moulting. It is clear from the results presented that this mobilization begins before the first morphological signs of premoult (initiation of new setae) appear. Moreover, we may conclude that the C_4 animals in the second sample were further advanced towards the premoult stage than were those in the first or third samples. There is no conclusive evidence that eyestalk removal has any effect on the mobilization of lipochromes.

The results of the carbohydrate determinations are presented in Table II. We may first note the rather striking difference in carbohydrate content of normal animals in stage C_1 between sample 2 and the other two samples. The mean values for samples 1 and 3 are 2.06 and 2.62 mg. per gm. for total carbohydrate, while the corresponding mean for sample 2 is 10.12 mg. per gm. The difference between the means for sample 1 and 2 is significant at the 5% level on the basis of the *t* test. This difference in means arises from the fact that all but one of the values from sample 2 are greater than 8 mg. per gm., while none of the values from samples 1 and 3 is as great as 5 mg. per gm. Moreover, the single low value in sample 2 was obtained from one of the animals (no. 26) which had no obvious lipochrome in the blood. If our earlier conclusion, that a substantial fraction of the animals in stage C_4 of the second sample were well on their way toward stage D_1, is correct, then we can further conclude that one characteristic of this transition is a marked increase in

TABLE II

Total carbohydrate and polysaccharide content (mg. glucose equivalent per gm. body weight) of three samples from a population of Carcinides maenas, and the effect of eyestalk removal

Stage condition	C_4						D_1					
	Normal			Eyestalkless			Normal			Eyestalkless		
	No.	Carb.	Poly-sac.	No.	Carb.	Poly-sac.	No.	Carb.	Poly-sac.	No.	Carb.	Poly-sac.
Sample 1	7	2.14	1.31	6	5.64	4.04	11	3.63	3.00	13	3.34	2.06
11–19 days	8	0.59	0.24	9	9.50	7.48	19	2.93	1.85	18	6.48	5.42
	12	2.38	1.70	10	8.59	7.67	20	6.10	5.24	21	6.19	5.05
	15	2.18	1.12	14	13.5	11.0						
	16	3.03	1.96	17	4.52	4.01						
Sample 2	22	8.88	8.48	24	9.45	9.20	31	4.83	4.83	35	15.7	13.9
43–45 days	25	12.7	8.57	27	9.53	9.53	32	16.4	16.4	41	5.45	5.25
	26	1.13	1.13	29	7.40	7.32	34	8.84	8.52	43	14.4	12.8
	28	15.7	14.8	30	12.8	12.3	40	14.4	13.7	23*	15.0	15.0
	36	12.2	11.6	33	16.5	15.8	38*	14.8	14.2	42*	23.6	10.9
				37	3.18	3.18						
				39	19.3	16.8						
Sample 3	45	1.36	1.36	44	8.13	8.13	47	1.45	1.45	48	1.72	1.41
67–70 days	49	2.73	2.21	46	9.40	7.06	55	9.41	8.48	50	3.52	2.98
	51	4.10	4.10	54	6.11	5.03	57	16.0	15.2	52	4.90	4.90
	53	2.28	1.88				59	12.8	11.1	56	12.7	11.0
							61	24.4	21.6	58	11.4	11.4
							63	15.7	11.9	60	2.49	1.95
							65	10.7	9.53	62	10.6	8.33
										64	18.9	16.3

* Stage D_2.

the carbohydrate content of the body. This conclusion is confirmed by the values for normal animals in stage D_1, which are nearly all well above those for the C_4 animals of groups 1 and 3. The difference between mean values for sample 3 for C_4 and D_1 is significant at the 1% level on the basis of the t test. Renaud (1949) had already observed a similar change in *Cancer pagurus* with a mean glycogen content of 2.09 mg. per gm. for animals in C_4, rising to 4.43 mg. per gm. by the end of D_1. We may therefore conclude that the increase in carbohydrate content which is characteristic of the transition from intermoult to premoult may occur during the latter part of stage C_4, before any morphological evidence of the transition is apparent.

TABLE III

Non-protein nitrogen (NPN) and ammonia nitrogen (NH_3N) in normal and eyestalkless Carcinides maenas (mg. per gm. body weight)

Stage condition	C_4						D_1					
	Normal			Eyestalkless			Normal			Eyestalkless		
	No.	NPN	NH_3N	No.	NPN	NH_3N	No.	NPN	NH_3N	No.	NPN	NH_3N
Sample 1	8	2.40	0.25	6	2.45	0.10	11	2.44	0.13	10	2.56	0.10
	12	2.89	0.17	9	2.52	0.08	19	2.84	0.05	13	3.07	0.12
	15	2.88	0.20	14	3.58	0.08	20	3.39	0.06	18	3.08	0.05
	16	2.58	0.07	17	2.65	0.08				21	3.82	0.04
Sample 2	22	2.90	0.16	24	2.68	0.24	31	2.91	0.19	35	2.96	0.30
	25	3.21	0.20	27	3.96	0.24	32	3.97	0.28	41	3.17	0.29
	26	2.03	0.13	29	3.22	0.23	34	3.13	0.24	43	3.30	0.25
	28	4.74	0.23	30	3.51	0.26	40	3.48	0.28	23*	3.17	0.32
	36	2.82	0.25	33	4.07	0.27	42*	3.69	0.42			
				37	2.72	0.19	38*	3.03	0.26			
				39	2.64	0.27						

* Stage D_2.

The second item to be noted from Table II is the fact that the carbohydrate content of the eyestalkless animals in C_4 is throughout at levels characteristic of D_1 animals. Indeed, there was no eyestalkless C_4 animal with a carbohydrate content as low as 3 mg. per gm., and in all but two, the value was higher than 5 mg. per gm. The differences in means for normal and eyestalkless animals in C_4 were significant at the 5% level for both samples 1 and 3, on the basis of the t test. We may therefore conclude that the operation of eyestalk removal causes an increase in carbohydrate content from the low values characteristic of C_4 animals to the higher values characteristic of the next stage in the cycle, D_1. The same operation is clearly without effect upon animals already in stage D_1 if for some reason these animals have low carbohydrate content, since there are several eyestalkless D_1 animals with relatively low carbohydrate values, and the distribution of values in normal and eyestalkless specimens in this stage is substantially the same. We may further infer from our results, though conclusive evidence is lacking, that some endocrine factor is secreted in the eyestalk during stage C_4, and that secretion of this factor stops towards the end of that stage. One effect of this factor would be the maintenance of carbohydrate content at relatively low levels. Since Renaud (1949) has shown a steady increase in glycogen content beginning in stage C_3, we may suppose that the secretion of the factor concerned decreases gradually rather than suddenly.

In general, the polysaccharide values follow the carbohydrate values rather closely, and 80% or more of the carbohydrate is precipitated by alcohol. However, in the C_4 animals of the first sample, the polysaccharide averages only 62% of the total carbohydrate; the eyestalkless individuals, and indeed all of the other groups, had a higher ratio. Blood carbohydrate was measured for the animals of sample 1 only. The results are presented in Table III. Since it appears that the carbohydrate content of the blood does not reflect changes in the total carbohydrate of the body, and is not influenced by any of the other factors considered here, we utilized the blood samples from the second and third group for lipochrome studies.

The observation of Needham (1955) that increased nitrogen excretion follows eyestalk amputation, led us to examine the nitrogen content of some of the extracts. The results are presented in Table IV. There appears to be no systematic variation in either NPN or NH_3N, except that both sets of values, and especially the NH_3N values, are generally lower in the animals of sample 1 than in those of sample 2. No obvious explanation for this difference appears. In both samples, the extracts were prepared 7 to 14 days after eyestalk removal, by which time Needham (1955) found that nitrogen excretion had returned to normal levels. We conclude that no long-lasting modification in nitrogen metabolism evident from NPN or NH_3N content of the animals is related to the variables considered here.

Discussion

Perhaps the most important finding of this study is that metabolic changes (mobilization of lipochromes, increased carbohydrate content) preparatory to the moult precede in time the morphological changes (formation of new setae). This may not be surprising, but it has not been emphasized before. We cannot on the basis of the evidence available conclude that the metabolic changes are causally related to the subsequent structural changes, but this is a reasonable inference. However, the two metabolic changes observed do not seem to be directly related one to the other. There is in general no complete correlation between increased blood lipochrome and increased carbohydrate. Moreover, the increase in carbohydrate which follows eyestalk removal is not in general associated with increased blood lipochrome.

The increase in carbohydrate content as the animal approaches a moult was already known from the study of Renaud (1949) on *Cancer pagurus*. Moreover, Schwabe *et al.* (1952) had observed a marked increase in total glycogen, represented by deposition in the digestive gland and epidermis, in the transition to the premoult stage in spiny lobsters; their data also suggest that eyestalk removal in stage C increases the total glycogen of the body, while the same operation in stage D results in no change. However, they did not determine this quantity directly, and neither of their methods, for determination of glycogen or for determining intermoult cycle stage, was entirely satisfactory. The demonstration of an increased carbohydrate content following eyestalk removal therefore comes as a definite addition to the long list of metabolic and other changes which are consequences of this operation (Knowles and Carlisle, 1956; Scheer, 1957).

The absence of any changes in blood carbohydrate was something of a surprise.

Renaud (1949) found a steady increase in the reducing power of the blood from C_3 through D_1 in *Cancer pagurus*, but this increase was not evident when the blood was treated with cadmium sulfate and sodium hydroxide, a procedure supposed to eliminate non-glucose reducing substances. Recent studies in my laboratory by McWhinnie (unpublished) on the blood of *Hemigrapsus nudus* have shown that the blood carbohydrate, like the total carbohydrate in acid extracts of the body, includes several components, of which glucose is a relatively minor one. Using the highly specific hexokinase glucose-6-phosphate dehydrogenase method, she found glucose concentrations averaging below 2 mg. per 100 ml., with a maximum about 2.5 mg% in stage C_1, and a slight decrease in stage C_3, but no change as a result of eyestalk extirpation. We had earlier found a decrease in the reducing substances of spiny lobster blood (Scheer and Scheer, 1951) following eyestalk removal, but others (Abramowitz *et al.*, 1944; Kleinholz and Little, 1949) found no such change in crabs. It is clear that the problem of blood sugar regulation in crustaceans requires further careful study with particular attention to specificity of methods.

The question now arises, what is the source of the increased carbohydrate in late intermoult, and what alterations in metabolism are responsible for the increase. Related to this is the question of the endocrine factors which we presume to be responsible for the increase. The evidence on which we can base hypotheses remains fragmentary. Injection of eyestalk extracts increases blood reducing substances, and specifically the fermentable reducing substances (Abramowitz *et al.*, 1944; Kleinholz and Little, 1949; Scheer and Scheer, 1951). It would be unwise at present to equate fermentable reducing substances with glucose, and we do not know the metabolic relations among the various carbohydrates found in the blood, nor indeed the identity of these substances. Scheer and Scheer (1951) showed that injected glucose was removed from the blood more rapidly in eyestalkless than in normal spiny lobsters, and that most of the carbon of this glucose could be recovered in the water- and alcohol-soluble fraction of tissue extracts. From the work of Hu (1958) we know that this fraction may contain, besides glucose, several oligosaccharides of the maltose series. But the relation of these substances to synthesis of polysaccharides or other aspects of carbohydrate metabolism remains obscure. Present evidence, from plants and animals alike, indicates that glycoside linkages in general are formed by adding one monosaccharide unit at a time to existing nuclei by the agency of nucleotide coenzymes. Hu (1958) has shown that nucleotides are present in crabs, and that carbon from administered glucose appears in these compounds.

Whatever the intermediate steps, the increased carbohydrate content of late intermoult crabs may be derived ultimately either from protein or carbohydrate or both. The evidence that, in fed crabs, there is no change in the non-protein nitrogen, suggests that there is no fundamental alteration in the intensity of protein metabolism. On the other hand, the evidence of Neiland and Scheer (1953) that, in fasting crabs, protein is used in preference to carbohydrate, and in eyestalkless crabs, the amount of protein used is greater, together with the evidence of Needham (1955) that under conditions (trauma) in which protein breakdown is increased eyestalk removal leads to a further increase, suggest that, in eyestalkless animals there may be an increased conversion of protein to carbohydrate. The fact that, in such animals, the utilization of glucose is also increased (Scheer and Scheer, 1951) would lead one to place the site of the presumed endocrine effect in the process of glycogenesis, rather than in that of gluconeogenesis. We may, therefore, postulate that, during the C_4 stage, there is a gradual transition from carbohydrate oxidation to polysaccharide synthesis as a major pathway of carbohydrate metabolism. This offers a possible explanation for the difference in glucose oxidation observed by Hu (1958) and Scheer and Scheer (1951) using similar procedures with different animals. The crabs used by Hu may have been in the early part of stage C_4 or even in C_3, when carbohydrate oxidation is dominant; the spiny lobsters used by Scheer and Scheer (1951) may have entered into the phase in which carbohydrate synthesis predominates. Neither author determined the intermoult stage with great accuracy. Or, if we accept the view of Carlisle (Knowles and Carlisle, 1956) that the intermoult cycle is qualitatively different in animals with prolonged intermoults (diecdysis, as in crabs in winter) from the intermoult in animals which moult regularly throughout the year (anecdysis, as in Hawaiian spiny lobsters), we might suppose that carbohydrate oxidation is primarily characteristic of diecdysis and that in anecdysis polysaccharide synthesis predominates. These suggestions can be tested by careful comparison of the fate of carbon from administered labeled glucose in the various stages of moult cycles of both types.

The question of hormonal control is likewise a difficult one. Carlisle (Knowles and Carlisle, 1956) has summarized evidence suggesting that two separate hormones are concerned in the control of the intermoult period. Carlisle attributes diecdysis to the action of the well-known moult-inhibiting hormone, and cites his own observations and some of ours (Scheer and Scheer, 1954) to the effect that this hormone is not active in certain crustaceans, including British populations of *Carcinides maenas* in the summer. We have no way of knowing whether the animals studied in the present investigation undergo a cycle of the type characterized by anecdysis, or one characterized by diecdysis. It would appear that, to make much further progress with these problems, it will be essential to work with at least partly purified hormone preparations, and to have full information about the type of cycle and stage of the animals in the cycle. We earlier postulated (Scheer and Scheer, 1951) an eyestalk factor which restrains carbohydrate utilization for polysaccharide, and specifically chitin, synthesis. This factor would be the same as the "diabetogenic" factor of Abramowitz *et al.* (1944), and we suggested that it might also be the moult-inhibiting factor. The results reported here do not give us any reason to alter this hypothesis, nor do they substantially strengthen it. However, at present it seems best to consider that the effects of eyestalk removal noted by Neiland and Scheer (1953) on protein catabolism, and the related effect noted by Needham (1955), might result from the action of this same factor. For conclusive evidence concerning this hypothesis, it is essential to have a hormone preparation of reasonable purity which can be tested for its specific metabolic effects.

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SUMMARY

1. A laboratory population of *Carcinides maenas* was sampled three times over a period of 70 days, and the blood carbohydrate, blood lipochromes, total body carbohydrate, total body polysaccharide, non-protein nitrogen, and ammonia nitrogen were determined; the effect of eyestalk removal on these quantities was also examined.

2. During the course of the observations, there was a progression within the population from late intermoult (C_4) to early premoult (D) stages.

3. The change from intermoult to premoult was signalized by the appearance of relatively large amounts of lipochromes in the blood and integument, and by an increase in total body carbohydrate content. These biochemical changes preceded any morphological signs of preparation for moult.

4. Eyestalk extirpation caused an increase in the body carbohydrate, but did not alter the blood lipochromes. The increase in carbohydrate was observed only in those animals which had not undergone the change spontaneously.

5. The other quantities measured showed no variation attributable either to the stage in the intermoult cycle or to eyestalk removal.

6. The results are discussed with relation to the possible mechanisms of the effects observed, and the hormonal factors concerned.

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THE LIFE-CYCLE OF THE DIGENETIC TREMATODE, PROCTOECES MACULATUS (LOOSS, 1901) ODHNER, 1911 [SYN. P. SUBTENUIS (LINTON, 1907) HANSON, 1950], AND DESCRIPTION OF CERCARIA ADRANOCERCA N. SP.

HORACE W. STUNKARD[1] AND JOSEPH R. UZMANN

U. S. Fish and Wildlife Service

The genus *Proctoeces* was erected by Odhner (1911) to contain *Distomum maculatum* Looss, 1901, from *Labrus merula* and *Crenilabrus* spp. at Triest. Odhner had found the parasite in *Blennius ocellaris* at Naples. One adult specimen from *Chrysophrys bifasciata* and two immature specimens from *Iulis lunaris* taken in the Red Sea, were described as a new species, *Proctoeces erythraeus*. Dawes (1946) listed *P. erythraeus* as a synonym of *P. maculatus* (Looss), but the species was recognized by Manter (1947) on the basis of six specimens he had collected from *Calamus calamus* and *Calamus bajonado* at the biological laboratory of the Carnegie Institution at Dry Tortugas, Florida. Several additional species have been described. Fujita (1925) reported a metacercaria from the Japanese oyster, *Ostrea gigas,* as a new species, *Proctoeces ostreae*. The paper was translated by R. Ph. Dollfus who noted (p. 57), "Il est à souhaiter que des recherches chez les poissons mangers de Lamellibranches, sur les côtes de la préfecture d' Hiroshima, permettent de découvrir des exemplaires complètement adultes de *Proctoeces ostreae* Fuj., chez lesquels l'extension des vitellogènes et les dimensions des oeufs puissent être observées avec précision; il sera alors possible de savoir définitivement si *P. ostreae* Fuj. doit ou non tomber en synonymie avec *P. maculatus* (Looss)." Yamaguti (1934) described *P. maculatus* from *Sparus aries, Sparus macrocephalus, Pagrosomus auratus*, and *Epinephelus akaara* in Japan. Several specimens from *Pagrosomus auratus*, which differed from *P. maculatus* in larger size, larger eggs, and trilobed ovary, he described as a new species, *Proctoeces major*. Yamaguti (1938) reported *P. maculatus* from *Semicossyphus reticulatus* and described a larva from the liver of the pelecypod mollusk, *Brachidontes senhausi,* as an unidentified member of the genus *Proctoeces*. Manter (1940) described *Proctoeces magnorus* from a single specimen found in the intestine of *Caulolatilus anomalus,* taken at Cerros Island, Mexico. Hanson (1950) identified two specimens collected from *Calamus* sp. at Bermuda by the late F. D. Barker as *Distomum subtenue* Linton, 1907, a species described originally from *Calamus calamus* in the same area. Comparison of these specimens with those from Tortugas identified by Manter as *P. erythraeus* established their identity, and *P. erythraeus* was suppressed as a synonym of *Proctoeces subtenue* (Linton, 1907). Hanson corrected the statement of Manter (1947), noting that it is the vitellaria, not the uterus, which never extends into the post-testicular region. Yamaguti (1953) predicated that *Xenopera* Nicoll, 1915 is a synonym of *Proctoeces*, and *Xenopera insolitus* from *Sparus australis* was listed as *Proctoeces insolitus* (Nicoll, 1915). Winter (1954) described *Proctoeces macrovitellus* from the intestine of *Cymatogaster aggregatus*, taken off the coast of southern California. It is notable that the final hosts of these trematodes are porgies and labroid fishes of temperate and warm seas; hard-mouthed, bottom forms that feed on mollusks.

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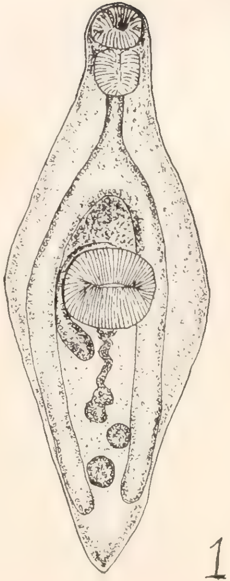
Uzmann (1953) described *Cercaria milfordensis*, a microcercous trematode larva from *Mytilus edulis* in both intertidal and subtidal areas of Long Island Sound and along the cost of Connecticut. About seven per cent of the mussels were infected in the years 1951 and 1952. Although the infection was heavy in the area around Milford, Connecticut, Uzmann noted that the parasite had not been reported from higher latitudes despite intensive study of *M. edulis* over a period of many years. The sporocysts develop in the venous sinuses of the mussel, beginning in the late fall and continuing during the winter, with the release of the cercariae in greatest numbers in the late winter and spring. The infection largely destroys the gonad of the host and development of the sporocysts precludes normal gametogenesis. The intensity of the infection seriously impairs the vitality of the mollusk and may be lethal under temporary or sustained periods of ecological conditions unfavorable to the host. Uzmann described the behavior of the cercariae and reported unencysted progenetic larvae referable to the genus *Proctoeces* in mussels harboring *C. milfordensis* infections. He stated (p. 449), "Morphological comparison of the two forms is favorable, and if the apparent relationship truly exists, an abbreviated life-cycle may be possible since the larval *Proctoeces* contain many eggs with well developed, motile miracidia. Experimental studies are projected and it is hoped that decisive information can be presented at a later date." Shortly thereafter, Uzmann was transferred to the Seattle, Washington Laboratory of the U. S. Fish and Wildlife Service.

Further significant information was provided by the work of Hopkins (1954) who described infection of the hooked mussel, *Brachidontes recurvus* (syn. *Mytilus recurvus*) taken in Barataria Bay, Louisiana by *Cercaria brachidontis* n. sp., a species so similar morphologically to *C. milfordensis* that their relationship was immediately apparent. *Cercaria brachidontis* develops in orange-pigmented sporocysts which completely destroy the gonad of the mussel. Immature cercariae have small, knob-like tails, similar to those of *C. milfordensis*, but they are not present on fully developed larvae. Hopkins referred the species to the family Fellodistomatidae but without generic designation.

After the text of this paper was written, the account by Freeman and Llewellyn (1958) appeared, announcing the discovery of the adult stage of a digenetic trematode in the renal organs of the lamellibranch mollusk, *Scrobicularia plana* taken from the mud-flats of the Thames estuary, at Chalkwell in Essex and Whitstable in Kent. The worms were identified as *Proctoeces subtenuis* (Linton, 1907) Hanson, 1950, a species which was known previously only as a parasite of the hind-gut of marine fishes belonging to the families Labridae and Sparidae, which occur chiefly in tropical and subtropical seas. The asexual generations were not discovered and since the adult stages had not been recorded from fishes of the English coast, the authors concluded that in British waters the life cycle had been abbreviated and restricted to invertebrate hosts. Possible methods were considered by which the parasite had been introduced. They reported (p. 446) that, "The eggs are enclosed



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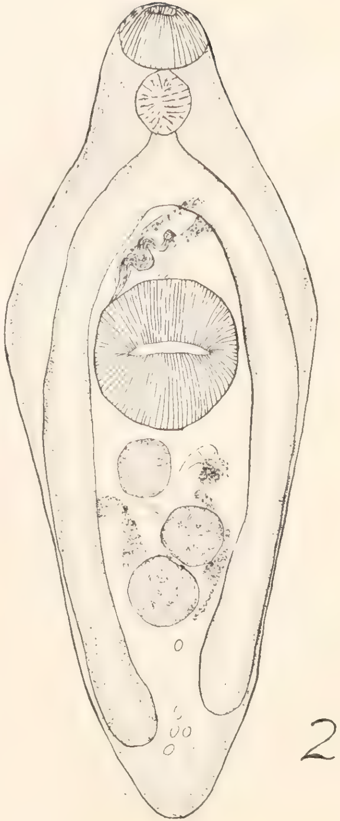

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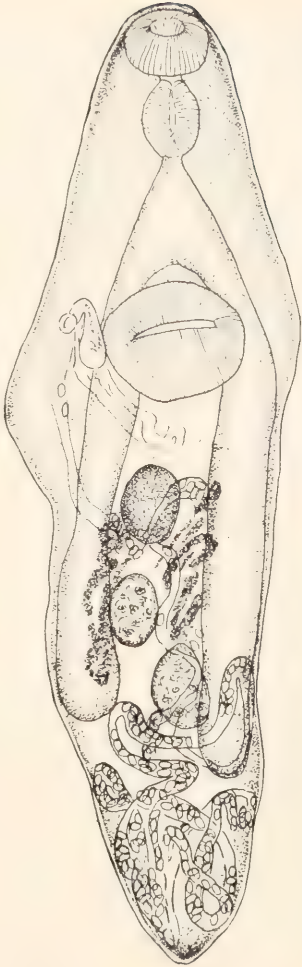

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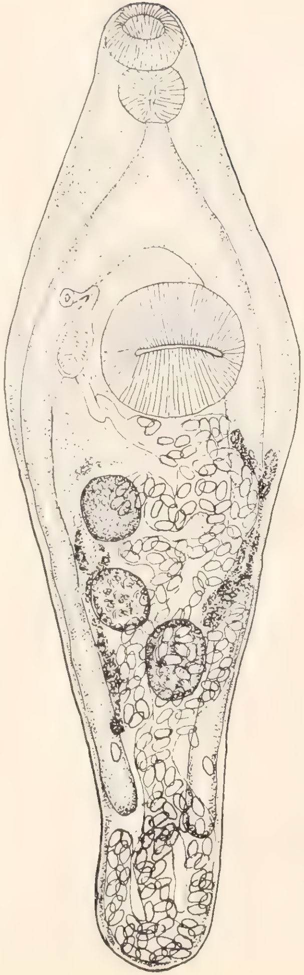


4

PLATE I

in a thin, light-brown capsule." This statement appears confusing, since the "capsule" is obviously the egg-shell and an egg comprises the shell and its contents, ovum, embryo, or miracidium. Although many eggs contained active miracidia, they varied much in size (from 0.026–0.073 by 0.015–0.030 mm.). The use of histochemical techniques disclosed the presence in the vitellaria of dihydroxy-phenols and protein, which on oxidation combine to form the quinones of the egg-shell, but the corresponding phenol-oxidase was not demonstrated. Deficiencies in the egg-making apparatus may account for the small and abnormal eggs. Freeman and Llewellyn gave a detailed account of the morphology of the parasite and noted the extent of individual variation. They stated (p. 447), "Several hundred specimens were examined, and it is apparent that many of the characters thought to indicate specific differences probably represent intraspecific variations of the kind emphasized by Stunkard (1957)." As a result of their investigation, *P. erythraeus* Odhner, 1911 and *P. magnorus* Manter, 1940 were suppressed as synonyms of *P. subtenuis*. Furthermore, they stated (p. 455) that, "The differences between *P. insolitus* (Nicoll, 1915) and *P. subtenuis*, and between *P. maculatus* (Looss, 1901) and *P. subtenuis*, require reexamination."

The findings of Freeman and Llewellyn amply confirm the postulate of Uzmann (1953) and constitute an important contribution to knowledge of the biology of the digenetic trematodes.

The studies begun by Uzmann at Milford were continued at Woods Hole, Massachusetts by the appointment of Stunkard to investigate the parasites of clams and their predators in New England. Infections by *C. milfordensis* were found in *M. edulis* taken in the Woods Hole area, although the incidence of infection was low, about 0.5 per cent. However, the findings of developmental stages, from cercariae to adults, confirmed the prescience of Uzmann that *C. milfordensis* is the larval stage of a species of *Proctoeces*.

DESCRIPTIONS

Adults. (Figs. 3, 4)

The general morphology of the worms is portrayed in the figures. The cuticula is unarmed; the suckers large and powerful. The digestive tract shows no unusual features. The excretory vesicle bifurcates at the level of the posterior testis; both the stem and crura are lined with a simple epithelium which is flattened when the wall is distended. The flame-cell pattern of the adult worm was not studied.

The genital pore is lateral, situated usually between the acetabulum and the pharynx. The testes are diagonally tandem, either adjacent or somewhat separated. Sperm ducts arise at the anterior ends, pass forward and join to form a common duct just before entering the cirrus sac. In the posterior end of the cirrus sac it forms a coiled seminal vesicle, filled with spermatozoa, and then opens into a straight, thick-walled muscular canal. This structure is lined with high, secretory cells, whose distal

PLATE I

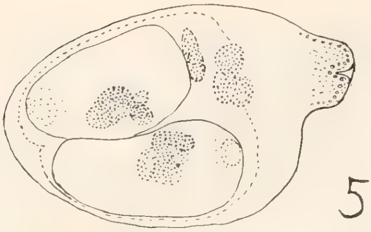
Drawings of *P. maculatus* from *M. edulis;* made from fixed and stained specimens and at the same magnification.

FIGURE 1. Juvenile specimen; length 1.20 mm.

FIGURE 2. Specimen just reaching sexual maturity, 6 eggs in uterus; length 2.00 mm.

FIGURE 3. Gravid specimen, eggs small and mostly misshapen; length 2.65 mm.

FIGURE 4. Gravid specimen, eggs normal with developing miracidia, length 2.62 mm.



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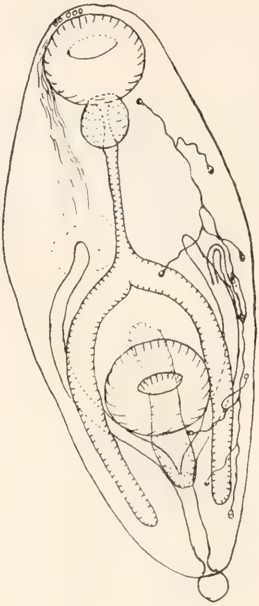

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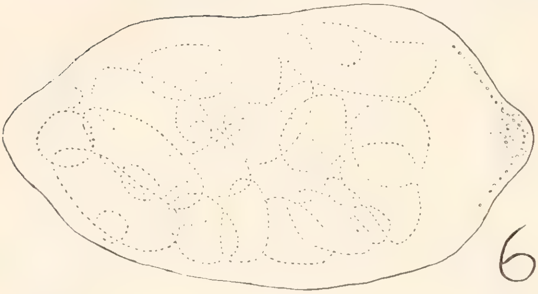

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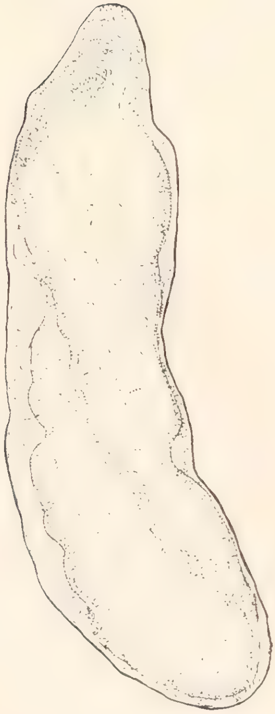

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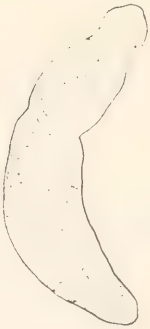

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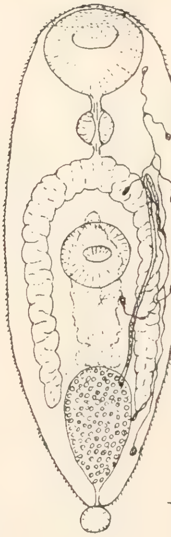

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Plate II

ends are filled with chromatic granules, and terminates in the cirrus which protrudes into a long, hermaphroditic atrial duct. The area between the wall of the cirrus sac and the thick-walled canal is filled with secretory cells, whose ducts pierce the thick wall and discharge into the narrow lumen. The ovary is pretesticular, in the anterior part of the posterior one-half of the body. The oviduct arises at its posterior face and turns ventrad and mediad where it expands into a fertilization space, from which Laurer's canal emerges and continues dorsad and anteriad to open at the surface above the ovary. The oviduct then enters Mehlis' gland where it receives the common vitelline duct and expands into the ootype, where the egg is formed. The uterus passes posteriad to the end of the body where coiled loops on either side are followed by a median trunk which passes forward below the cirrus sac to open into the ventral side of the hermaphroditic duct, six to ten microns before the genital pore. In many of the specimens the eggs are malformed, of varying sizes, often about one-third as large as in more normal individuals.

Average measurements in millimeters of ten gravid, mounted specimens; limits in parentheses: length, 2.74 (2.4–3.2); width, 0.81 (0.6–0.92); acetabulum, 0.38 × 0.43 (0.35–0.46); oral sucker, 0.24 × 0.30 (0.21–0.32); pharynx, 0.18 (0.16–0.20); ovary, 0.19 (0.16–0.22); anterior testis, 0.18 (0.15–0.20); posterior testis, 0.19 (0.16–0.23); eggs, 0.055 × 0.026 (see text).

Juveniles. (Figs. 1, 2)

Figure 2 shows a specimen just reaching maturity, which has 6 eggs in the uterus. It is somewhat flattened as a result of pressure during fixation. Measurements in millimeters are: length, 2.00; width, 0.80; acetabulum, 0.34 × 0.37; oral sucker, 0.18 × 0.22; pharynx, 0.125 × 0.150; ovary, 0.15 × 0.14; anterior testis, 0.17 × 0.15; posterior testis, same size.

Figure 1 shows a smaller and less mature specimen, also flattened during fixation. The acetabulum is almost exactly in the middle of the body; the post-acetabular region increases relatively in size with the development of the reproductive organs. Measurements are: length, 1.2; width, 0.56; acetabulum, 0.21 × 0.275; oral sucker, 0.128 × 0.15; pharynx, 0.125 × 0.125; ovary, 0.057; anterior testis, 0.079 × 0.072; posterior testis, 0.092 × 0.079.

Sporocysts and Cercariae. (Figs. 5, 6, 7, 8)

Descriptions of the sporocysts and cercariae were given by Uzmann (1953). His observations have been confirmed and additional data are presented. There are at least three generations in the mollusk. Figure 5 shows a sporocyst with two

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Plate II

Figure 5. *P. maculatus,* mother sporocyst with daughter sporocysts containing germinal cells of the next generation; length 0.34 mm.

Figure 6. *P. maculatus,* daughter sporocyst with developing cercariae; length 0.54 mm.

Figure 7. *P. maculatus,* large daughter sporocyst with developing cercariae, *Cercaria milfordensis,* length 1.18 mm.

Figure 8. *P. maculatus,* cercaria, from stained and mounted specimen, excretory system added from sketches of living worms; length 0.26 mm.

Figure 9. *Cercaria adranocerca* n. sp., daughter sporocyst from *G. gemma;* length 0.48 mm. This drawing is at the same magnification as Fig. 7.

Figure 10. *Cercaria adranocerca* n. sp., stained and mounted specimen, excretory system added from sketches of living worms; length 0.21 mm.

daughter sporocysts and in each of them there are heavily staining germinal cells of the next generation. The cercariae are subcylindrical and taper toward anterior and posterior ends. In addition to the three pairs of cephalic gland ducts reported by Uzmann, two additional pairs are sometimes visible; the cell bodies are lateral, in the preacetabular area, but so far it has been impossible to demonstrate them with certainty, either by the use of vital dyes or in permanent preparations. The flame-cell pattern has been worked out and is shown in Figure 8. On either side a duct, which contains patches of cilia, emerges from the excretory vesicle near its anterior end; it passes forward, loops backward and here receives the two collecting ducts. The anterior one of these ducts receives the fluid from four flame-cells and capillaries located in the anterior quadrant of the body; the posterior one from flame-cells and capillaries in the posterior quadrant. The flame-cell formula is $2[(2+2)+(2+2)]$.

Cercaria adranocerca n. sp. (Figs. 9, 10)

In addition to the specimens from *M. edulis*, described above, dissection of some three hundred *Gemma gemma* from the region of Boothbay Harbor, Maine, in August and September 1957, disclosed two infections by sporocysts and microcercous cercariae, similar to those from *M. edulis* and from *Brachidontes recurvus*. The sporocysts were relatively few, oval to sausage-shaped; the largest one measured 0.42 mm. long and 0.11 mm. wide after fixation while smaller ones, no larger than a cercaria, contained a few germ-balls. The end that bears the birth-pore may be extended as a long, tapering protrusion.

The cercariae were studied alive, unstained and after staining lightly with Nile blue sulphate and with neutral red; also after fixation as stained and cleared permanent mounts. Frequently, one adhered to the slide by the posterior end and extended the body in all directions. The body is subcylindrical, typically more rounded anteriorly than posteriorly.When extended, contraction of the circular muscles may produce an annulate appearance. The body wall is relatively strong for so small a larva. The cuticula bears rows of closely set, flattened spines. Ducts of cephalic glands were sometimes visible in the region of the oral sucker, but their number and the location of the cell bodies were not determined. Alive, cercariae measured from 0.16 to 0.33 mm. in length and 0.044 to 0.09 mm. in width. The tail is terminal, spherical, and measures 0.01 mm. in diameter; it is easily detached. The oral opening is subterminal, the sucker is 0.032 to 0.043 mm. in diameter. When the anterior end of the body is extended, the prepharynx is about one-half the length of the pharynx which measures 0.014 to 0.018 mm. in diameter. The ceca are relatively long, extending about midway between the acetabulum and the posterior end of the body. The acetabulum is situated in the posterior portion of the anterior half of the body and protrudes, although it is not stalked. It measures 0.025 to 0.036 mm. in diameter; the ratio in size between the oral and ventral suckers is about 4:3; although the size increased greatly over the figures given above when the cercaria was subjected to extreme pressure for the study of the excretory system. The region between the excretory vesicle and the acetabulum contains cells which stain deeply; they are the rudiments of the reproductive organs. The excretory pore is terminal, at the base of the tail; the vesicle is oval and may extend forward more than half the distance to the acetabulum. On either side, from the anterior end of the vesicle, a collecting duct passes forward to the level of the bifurcation of the digestive tract where it turns backward; the recurrent portion contains tufts of cilia and near the level of the acetabulum it receives anterior and posterior collecting tubules. The arrangement is portrayed in the figure and the flame-cell formula is $2[(2+2)+(2+2)]$.

In flame-cell formula and vestigial tail, this species is similar to *Cercaria milfordensis* and *Cercaria brachidontis*. In form of the excretory vesicle and presence of cuticular spines it more closely resembles *C. brachidontis* and although neither can be included in the genus *Proctoeces*, it is probable that they are larvae of some member of the family Fellodistomidae. The species, described as new in this paper, is designated *Cercaria adranocerca* (*adrano*, inactive, feeble).

Type and paratype specimens are deposited in the U. S. Nat. Museum, Helminth. Collection, No. 56236.

DISCUSSION

The discovery of unencysted metacercariae and of developmental stages from cercariae to gravid adults demonstrates that *C. milfordensis* is the larval stage of a species of *Proctoeces*. However, the progenetic worms are often not entirely normal. In some of the specimens (Fig. 3), the eggs are misshapen and of varying sizes, often not more than one-third as large as in other individuals. A similar situation was reported by Freeman and Llewellyn (1958). It appears that the female organs, especially the vitellaria, may be deficient or that the ova are not fertilized, and such abnormal eggs do not contain miracidial larvae. For this reason, the extent and development of the vitelline follicles may not provide sound data for specific criteria.

Identification of these specimens presents disturbing problems. The description of *P. maculatus* by Looss (1901) is based on the largest of his specimens and is illustrated by a good figure. The characterization of *P. erythraeus* was very inadequate; Odhner gave no figure or measurements and the species was distinguished from *P. maculatus* because in the single mature specimen the acetabulum was one-third smaller, the eggs were smaller, and the vitellaria did not extend as far posteriad, a condition which might be expected in a specimen just reaching maturity. For this reason, Dawes (1946) suppressed *P. erythraeus* as a synonym of *P. maculatus*. The six specimens taken by him from *Calamus* spp. at Tortugas agreed with Odhner's account and Manter (1947) recognized *P. erythraeus* as a valid species, but there was no figure and as yet there is no complete description of *P. erythraeus*. In the (1947) paper, Manter stated that his (1940) listing of *Proctoeces* and *Tergestia* in the family Monorchidae was an error, since the family name, Fellodistomatidae, was accidentally omitted.

In a report on parasites of Bermuda fishes, Linton (1907) published the description of a new species, *Distomum subtenue*, from *Calamus calamus*. Smaller, immature specimens were found in other hosts, two in *Iridio bivittatus*, and one each in *Harpe rufa* and *Lachnolaimus maximus*. Although two small specimens are reported on p. 106 from *H. rufa*, the table on p. 87 shows that only one trematode was found in this host. In the "Food notes" on the fishes, which accompanied his account of their parasites, Linton stated that *C. calamus* feeds on mussels and crabs; the others on mollusks, crabs, sea urchins and annelids. All are bottom feeders and Breder (1929), in describing these fishes, stated that the mouths of porgies (*C. calamus* is the saucer-eye porgy) are (p. 180), "armed with strong jaw teeth," and that the members of the Labridae are (p. 202), "usually provided with strong canine teeth. . . . These fishes are provided with powerful pharyngeal teeth with which they crush mollusks."

Comparison of Linton's description and figure of *Distomum subtenue* with the two specimens from *Calamus* sp. taken at Bermuda by Barker and the six specimens taken from *Calamus* spp. at Tortugas by Manter, led Hanson to the conviction that all were conspecific and accordingly she (1950) announced the specific identity of *Dist. subtenue* Linton, 1907 and *P. erythraeus* Odhner, 1911. The species was designated *Proctoeces subtenue* (Linton, 1907). Again, there was only a scanty description and no figure. Manter (1954) identified five specimens from *Latridopsis ciliaris*, taken near Wellington, New Zealand, as *Proctoeces subtenue* (Linton, 1907) Hanson, 1950, and listed the species from the Red Sea, Bermuda, Tortugas, and New Zealand. If this determination is correct, the parasite is widely distributed and infects different kinds of fish. The latter point is probably not significant since the worms are progenetic and young mature specimens could be taken from the digestive tract of any fish which had recently ingested an infected host-mussel. Dollfus (in Fujita, 1925) was undoubtedly correct in the prediction that mollusk-eating fishes would be found to harbor the adult stage of *Proctoeces ostreae*, the unencysted metacercaria discovered by Fujita. Since members of the genus *Proctoeces* develop and may actually mature in bivalve mollusks, it seems certain that fishes may acquire the infection by eating these mollusks, although another method is of course not precluded.

Linton's (1907) description of *P. subtenuis* is accompanied by a figure and although done over fifty years ago it was, until the paper by Freeman and Llewellyn (1958), the most complete account of the species available. The length and width of the specimens and the sizes of the oral and acetabular suckers as given by Linton are actually greater than the corresponding measurements given by Looss (1901) for *P. maculatus*. Although *P. subtenuis* may be specifically distinct, there is at present no adequate basis for distinguishing between it and *P. maculatus*. The progenetic specimens described in the present paper are almost certainly identical with those described by Linton, and until they can be distinguished from *P. maculatus,* should be assigned to that species.

Proctoeces is clearly a member of the family Fellodistomidae, the name of which was confirmed in a letter by the late Charles W. Stiles and published in Stunkard and Nigrelli (1930). Cable (1953) recognized four subfamilies: Fellodistominae Nicoll, 1909; Gymnophallinae Odhner, 1905; Haplocladinae Odhner, 1911; and Tandanicolinae Johnston, 1927. Dollfus (1947), however, had maintained that *Monascus* Looss, 1907 has priority over *Haplocladus* Odhner, 1911 and that the correct name of the subfamily is Monascinae. Finally Freeman and Llewellyn (1958) pointed out that the excretory vesicle in members of the genus *Proctoeces*, which has an epithelial lining, controverts the thesis of La Rue (1957) that in the Anepitheliocystidia, in which the family Fellodistomidae is included, the definitive bladder is not epithelial.

ABSTRACT-SUMMARY

Sexually mature worms from *Mytilus edulis,* taken in Connecticut and Massachusetts, are identified as *Proctoeces maculatus* (Looss, 1901). The specimens are often sterile, which reflects the abnormal conditions of development in the molluscan

host. Similar worms were reported by Freeman and Llewellyn (1958) from *Scrobicularia plana* taken in the Thames estuary, England, and identified as *Proctoeces subtenuis* (Linton, 1907), but we regard *P. subtenuis* as identical with *P. maculatus*. Evidence is presented to show that *Cercaria milfordensis* Uzmann, 1953 is the larval stage of *P. maculatus*. The taxonomy of the species is discussed. *Cercaria adranocerca* n. sp. is described from *Gemma gemma* taken at Boothbay Harbor, Maine. It is not congeneric with *P. maculatus*, but is referred tentatively to the family Fellodistomidae.

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TWO NEW GENERA OF DINOFLAGELLATES FROM CALIFORNIA[1]

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The coastal waters in the San Diego region support fairly abundant populations of phytoplankton. Armored dinoflagellates of this region were studied extensively by Kofoid and his associates (1907–1933), but there are still numerous undescribed or little known representatives especially among the smaller species. In the present paper, two new genera and species are described. These were originally isolated by Dr. Beatrice M. Sweeney in 1956–57 from coastal water at La Jolla, Calif., and have since been maintained as laboratory cultures.

Acknowledgment is here made to Prof. Francis T. Haxo and Prof. Martin W. Johnson for their interest and for providing research facilities. The author is indebted to Dr. Beatrice M. Sweeney whose cultures made this study possible, to Mrs. Anne Dodson for valued technical aid and to Dr. K. A. Clendenning for assistance in the preparation of the manuscript.

METHODS

The dinoflagellates were first examined alive. Fixed material was then studied under an oil immersion objective and by phase contrast. To derive the general plate formulae of the thecae, an individual cell was isolated under a cover-glass. A drop of concentrated sodium hypochlorite solution was then passed slowly under the cover-glass to destroy the protoplasm and to remove the cement which unites the plates. This process was assisted by applying very gentle pressure to the cover-glass, but great caution was necessary because of the fragility of the specimens. With *Scrippsiella*, it proved helpful to store droplets of the cultures in a wet chamber for a few hours. Under these conditions many of the cells shed their thecae, to which the hypochlorite treatment was then applied. After testing other methods, the following technique was adopted for the examination of *Fragilidium*. Actively swimming individuals were killed by transferring them into 5% formaldehyde with a micropipette. Individual specimens were next isolated, and by applying gentle pressure to the cover-slip, the protoplasm was

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[1] Contribution from the Scripps Institution of Oceanography, New Series.

[2] Permanent address: Casilla de Correo 64, Necochea, Argentina. This work was conducted during the tenure of a John Simon Guggenheim Fellowship, 1957–58, at the Scripps Institute of Oceanography, University of California, La Jolla.

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Scrippsiella sweeneyi n. gen., n. sp.

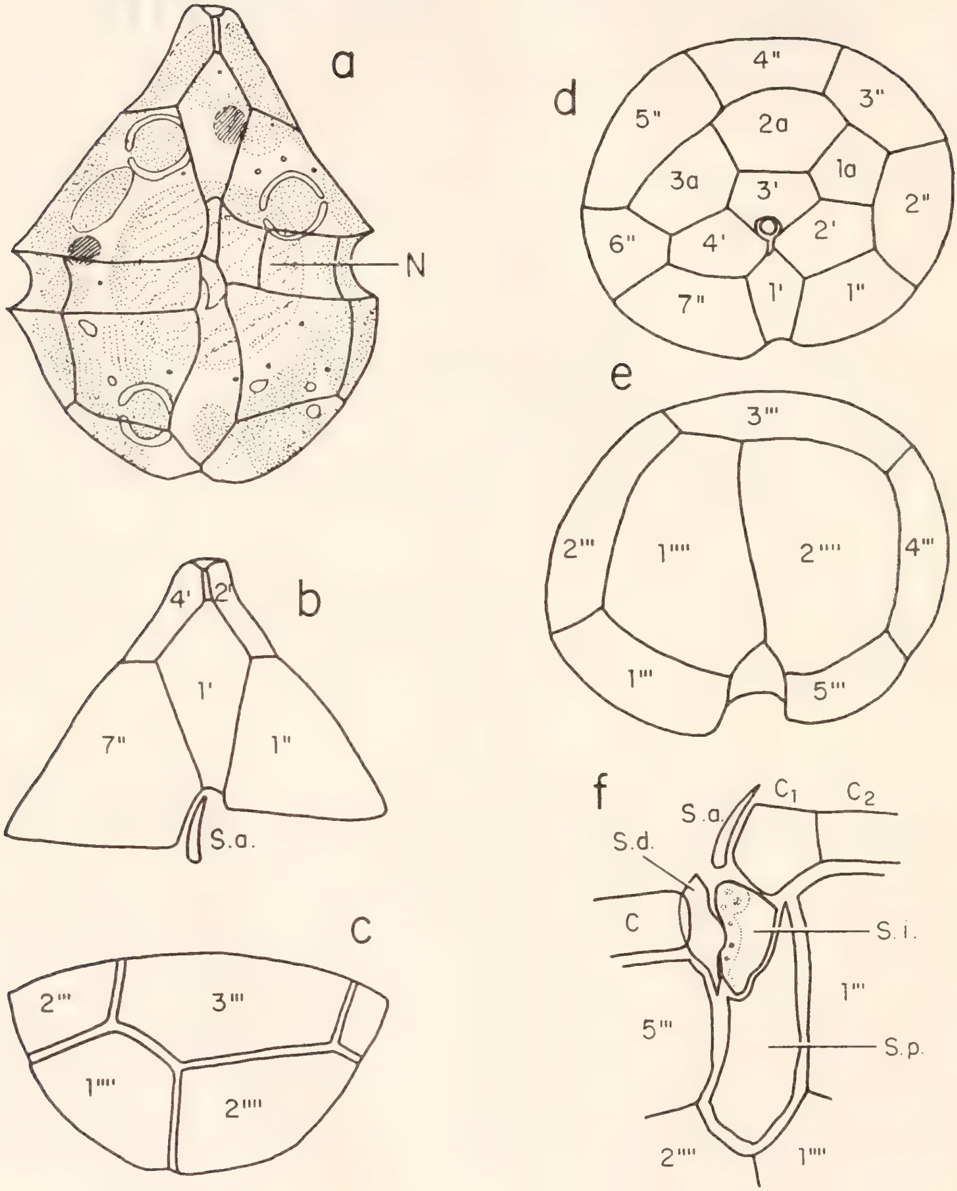
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FIGURE 1. *Scrippsiella sweeneyi.* a) A typical individual, ventral view. b) Ventral view of the epitheca. c) Dorsal view of the hypotheca. d) Apical view of the epitheca. e) Antapical view of the hypotheca. f) Sulcal region (S.a.: Anterior sulcal.—S.i.: left sulcal.—S.d.: right sulcal.—S.p.: posterior sulcal). All figures about × 1500.

forced out of the theca through the cingular region. The hypochlorite treatment was then applied to the empty *Fragilidium* theca, especially in studies of the sulcus and cingulum.

Diagnosis. Small-sized, conical epitheca, rounded hypotheca, without horns. Cingulum wide, cavazone, descendent, with displacement equal to two-thirds of its width, without lists. The cingulum has six plates, five equal, preceded at the left by a transitional one. Sulcus deep, of medium width, slightly curved to the right.

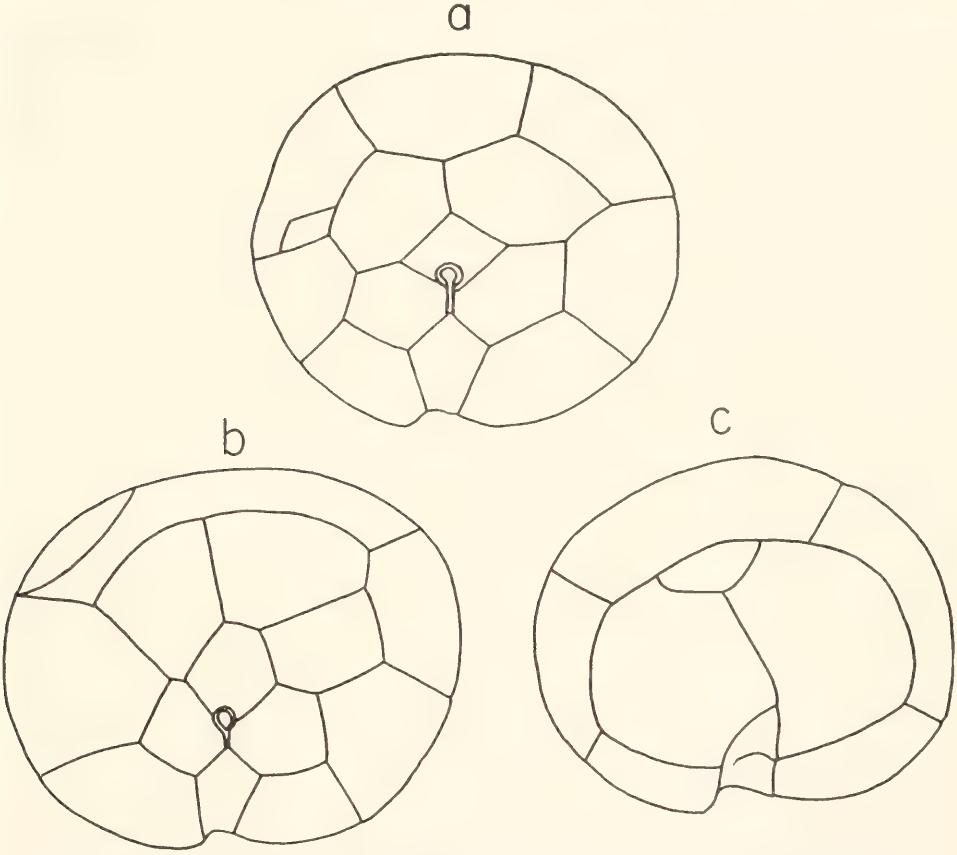

FIGURE 2. *Scrippsiella sweeneyi,* atypical plate patterns. a and b) Two epithecae, apical view. c) Antapical view of a hypotheca. All figures about × 1500.

The sulcus has four plates, with the posterior plate largest. The pattern of the major body plates is the same as that of an *Orthoperidinium* with three intercalaries. Cell length, 24–32.5 μ; transdiameter, 19–24 μ, chromoplasts numerous, elliptical, generally brown-yellow. La Jolla, California.

Description. This organism resembles *Peridinium trochoideum* in its general shape and size, and to some degree in its plate formula: 4′, 3a, 7″, 6c, 5‴, 2⁗, and 4s. Its epitheca is high and conical, most individuals deviating from a rectilinear outline by a concavity near the apex, as shown in Figure 1a. The hypotheca is almost hemispherical, and slightly shorter in length than the epitheca. In the region of the girdle, there is a slight dorsiventral compression. In apical view, the cells normally appear almost circular. The sulcus indents slightly into the epitheca, is very deep, and of medium width. It does not reach the antapex when in true frontal view.

The plate pattern of the major body plates is the same as that of an *Orthoperidinium* with three intercalaries. In the epitheca, the first apical plate (1′) is very narrow, with an asymmetrical rhombic shape and upwardly curved base. Attached to its anterior end, there is an extremely narrow ventral apical plate. The apex of the theca is horizontal, and is closed by a circular plate (apical pore platelet) which indents the pentagonally shaped third apical plate (3′). Plates 2′ and 4′ are comparatively large, and generally 2′ is a little wider than 4′. There are three dorsal intercalaries. Plate 2a is usually pentagonal but is sometimes hexagonal.

In the hypotheca, there are five post-cingulars and two antapicals. Plates 1″′ and 5″′ are wide, and 3″′ is very asymmetrical; its border with 2″″ is very long in comparison with its border with 1″″. The two antapicals have a very restricted connection with the end of the sulcus.

The cingulum has five plates of similar size, plus a transitional plate at the left end which is somewhat different in shape and also a little higher than the other cingular plates.

The sulcus of dinoflagellates is not easily examined, and has been neglected by most protistologists for that reason. The sulcus of *Scrippsiella sweeneyi* is exceptionally difficult to analyze, being about as difficult to study as that of *Heterocapsa triquetra*. The anterior sulcal plate (S.a.) is narrow and a little curved. It borders 7″. Posterior to this plate are two smaller plates (S.i. and S.d.). The shorter and broader of these two is the left plate (S.i.), which extends very slightly beyond the distal end of the girdle. The right border of this plate (S.i.) is thickened and refringent; it is provided with poroids and at the extreme anterior end there are two closely spaced pores. The right sulcal plate (S.d.) narrows toward the posterior. The posterior plate (S.p.) is the largest, forming the greatest part of the sulcus. Its right anterior border is strongly oblique to the axis of the plate and articulates with S.i. The posterior right border of S.p. is thickened.

The nucleus is round and located at the girdle level. Its diameter is about one-third of the total cell length. The chromatin strands are less evident than in most dinoflagellates. The chromoplasts are elliptical and numerous, sometimes yellow-green but normally brown-yellow. Food is apparently stored as small granules and also around the chromoplasts in bodies that resemble pyrenoids. There is no pusule nor stigma.

The first external evidence of cell division is the formation of two discrete longitudinal flagella with separate points of attachment. During division, the cell escapes from the theca but retains a tough cellular membrane. The two daughter cells remain attached to each other in an oblique plane. The posterior cell is usually the smallest.

Locomotion is normally rapid, with a strongly rotatory motion. There is usually one complete rotation of the organism during an advancement of one or two cell lengths. Sometimes, when *S. sweeneyi* cells reach the border of a drop, they suddenly cast off their flagella. Generally they lose the transverse ribbon-like flagellum first, which continues to beat in the detached state for a few seconds and then vacuolizes. The longitudinal flagellum is about three times as long as the cell; it does not beat or vacuolize after detachment.

Occurrence. This organism was originally isolated on March 15, 1956, from water collected off the S.I.O. pier at La Jolla, California, and has since been observed frequently in locally collected water samples. It seems to be a year-round inhabitant of the San Diego region, thriving especially in the summer months. This species has also been observed in plankton net samples, its relative scarcity in these being caused by its small size and poor retention on plankton silk.

Variations. The cingular and sulcal formula has been constant in laboratory and field specimens: 6C and 4S. The cells varied in size, and in the laboratory clonal culture used in the description, the cells also varied in shape and in plate pattern. Deformed or aberrant forms of *S. sweeneyi* were numerous in old laboratory cultures, but these were not observed in plankton samples. The plate formulae of the thecae from plankton samples were established in only a few cases, so we do not yet know how much the plate formula of this organism varies in nature. On the whole, the plate pattern has shown an amazing range of variation.

The normal plate formula is as stated above: 4′, 3a, 7″, 6c, 5‴, 2⁗, and 4s. It is generally assumed that the hypotheca is more conservative than the epitheca, and this is true of the present organism. Deviations from the normal epithecal plate configuration were observed in about 10 per cent of the specimens examined. The range of variation encountered in the atypical specimens of *S. sweeneyi* was rather exceptional for dinoflagellates, although similar variations occur in *Pyrophacus horologicum.* Plate formulae in these atypical specimens were:

(1) 4′, 2a, 6″;
(2) 4′, 3a, 5″;
(3) 4′, 3a, 4″;

A single specimen with 3′, 3a and 5″ showed an exceptional overgrowth of 1″, which reached the apical pore thus transforming 2′ into 1*a*.

No alteration of hypothecal formula has been noticed in actively growing cultures. In old cultures, I have observed hypothecal formulae of: 5‴, 2⁗ and one intercalary; 4‴, 2⁗; and 3‴, 2⁗ and one intercalary. However, the plate variation of the hypotheca has not exceeded two per cent in all examined specimens.

Discussion. The general characteristics of this organism place it in the *Peridiniaceae.* If it were classified solely on the basis of its major body plates, it would be included in the genus *Peridinium.* The cingular formula and sulcus plates are characteristically different, however, from those of *Peridinium.* The cingular and sulcal plates are conservative and important structural features connected with the most dynamic parts of the cell. Undoubtedly this organism belongs to a new genus. The species is also new. The only other known species which bear general resemblances to the present organism are *Peridinium subsalsum* Ost., and especially *P. trochoideum* (Stein) Lemm. Laboratory cultures of these species were provided by Dr. Sweeney for comparative studies. Their assignment to the genus *Peridinium* was clearly correct, but they bore only superficial resemblances to *Scrippsiella sweeneyi*. The genus is named after the institution at which it was discovered, and the species is dedicated to Dr. Beatrice M. Sweeney who made the original isolation and whose cultures made this study possible.

Fragilidium heterolobum n. gen. n. sp.

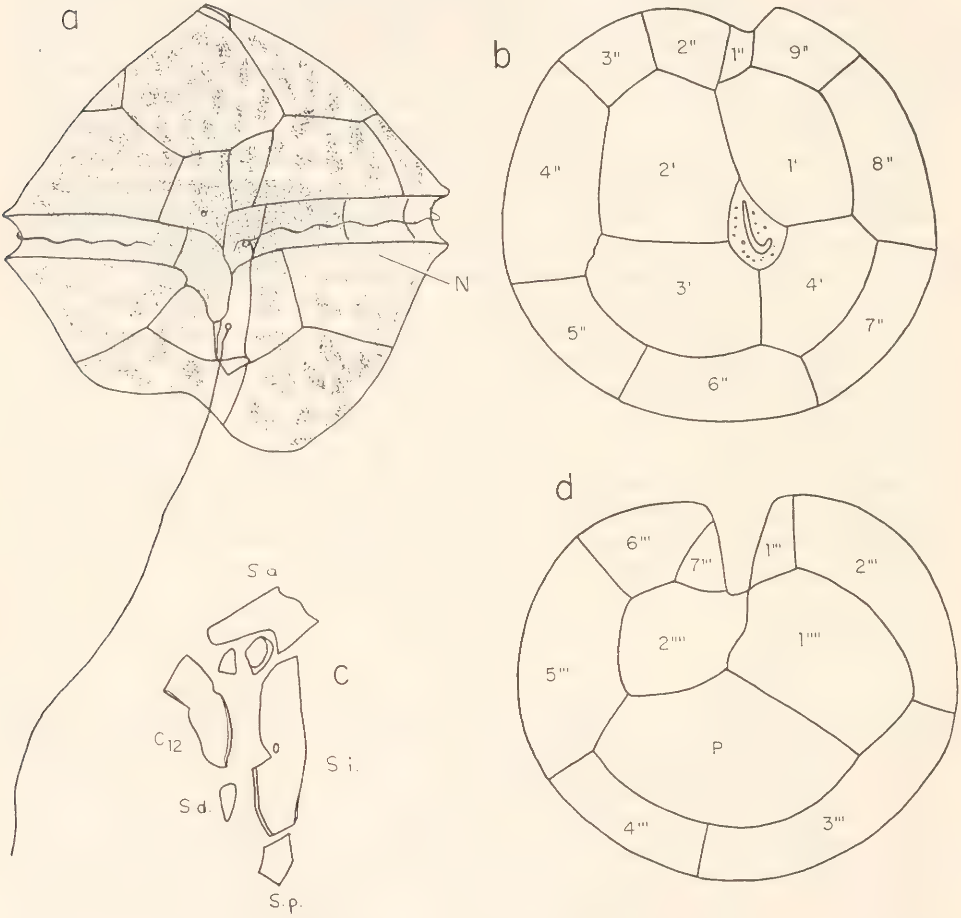

FIGURE 3. *Fragilidium heterolobum*. a) A typical individual, ventral view. b) Apical view of the epitheca. c) Sulcal plates. d) Antapical view of the hypotheca. All figures about × 1000.

Diagnosis. Medium-sized, roughly roundish pentagonal in ventral view. Epitheca dome-shaped; hypotheca asymmetrically bipedal, the left lobe being the largest. Cingulum deeply impressed, subcentral, descending, displaced distally about one girdle width, without lists. The cingulum has eleven sub-equal rectangular plates plus a transitional plate at the right end. Sulcus narrow, only slightly excavated, with six plates. Theca easily exuviated. Cell length 53–56 μ, transdiameter 48–54 μ. Chromoplasts numerous, elliptical, brown. Genus characterized by the high number of precingular, postcingular and cingular plates. La Jolla, California.

Description

Plate pattern. The epithecal formula is 4′, 9″ and a "pore platelet." The plate 1′ is in general large and it has the most irregular form. It has connections with seven plates: 1″, 2″, the pore platelet, 4′, 8″ and 9″; its border for 2″ is the smallest. Plate 1′ is asymmetrically located, most of it being on the right side of the epitheca; its width decreases gradually to the left. The other three apicals are more regular. Apical 2′ has six edges (for 1′, 3′, the pore platelet, 2″, 3″, 4″). The plate 3′ touches 2′, 4′, 4″, 5″, 6″ and the pore platelet. The apical 4′ touches 3′, 1′, 6″, 7″, 8″ and the pore platelet.

The so-called pore platelet is relatively large, oval sigmoid, and placed obliquely, to the median plane, *i.e.*, the plane which passes through the sulcus, the joint of 1″ and 9″, and the apex. This plate is variable but generally it has a convex left side subdivided into two edges for 2′ and 3′, a concave side which touches 1′, a major pole for 4′, and a minor one for the suture between 1′ and 2′. The most characteristic feature of this plate is a long and narrow reinforcement at the middle of the plate, sigmoid, with a dorsal hook to the right; it is variable sometimes double. Along it there are sometimes a few very small pores.

The most characteristic precingulars are 1″ and 9″. The first, trapezoidal in shape, is the smallest. The precingular 9″, pentagonal, has two edges at the left: the superior one for 1′ and the posterior, reinforced, forms a part of the right border of the sulcus. The precingulars 3″, 5″, and 7″ are more or less quadrangular.

The hypothecal formula is 7‴, 2⁗ and 1p. The narrowest postcingular plates are 1‴ and 7‴. The latter is the smaller and is somewhat displaced posteriorly. The antapicals are very asymmetrical, the left one being much longer. The suture between 1⁗ and 2⁗ is irregular. Antapical 2⁗ contacts the sulcus more than 1⁗, which just barely touches it. The intercalary (p) is a large irregular plate, bordered by the antapicals, 3‴, 4‴ and 5‴.

All plates of the epi- and hypotheca are smooth. Some spots of different optical densities could be seen in a few specimens, especially in plate 1″, with oil immersion and phase contrast. There are sometimes pores located on the cingular border of this plate.

The cingulum is formed by eleven subequal retangular plates, plus another different plate at its right end. This C_{12} is curved, irregular, extending somewhat into the sulcus, with a narrow left-posterior or sulcal end. For that reason this plate could be named "transitional." The cingular plates lack sculptures.

The sulcus is narrow, has six plates, and is only slightly excavated. The anterior sulcal plate has a very characteristic "boomerang" shape, with a posterior concavity and a longer and narrower right arm. In its sinus there are two very tiny platelets; the right one is the smaller. Behind the anterior plate and in contact with 1‴ there is a long plate, with a little sinus at the middle of its right border, where C_{12} ends. In connection with the latter, there is another small plate. Finally, there is a posterior sulcal plate.

Protoplasm. The protoplasm is surrounded by a strong membrane and contains more than one hundred elongate-elliptical chromatophores which are dark yellowish-brown. Food is stored as numerous granules of variable shape, which are generally small and located most abundantly in the peripheral layer.

The nucleus is large and compact, and is surrounded by a strong membrane. It is elongated in the equatorial plane, is somewhat curved, and has very dense thin threads of granular chromatin more or less perpendicular to the major axis of the nucleus. At the concavity, I sometimes observed large masses that were not distinctly granular.

The longitudinal flagellum extends beyond the antapex about two and a half cell lengths; it has a fast vibratory movement of short amplitude. The transverse flagellum, very slightly flattened, is long and completely encircles the girdle. The organism swims with a predominantly rotating motion.

Dimensions (in fixed and slightly distorted cells). Length 53–56 μ; transdiameter 48–54 μ. In an individual with a length of 55.5 μ, the epitheca was 27.5 μ and the hypotheca was 24 μ in length.

Variations. I have observed some variation in form (cell length more or less short in comparison with the transdiameter) and also in the plate pattern. Sometimes 4″ appears divided into two plates; thus the postcingular series sometimes has eight instead of seven plates. Occasionally there are eight instead of nine precingulars, and in one individual, a very narrow 1′ was observed fused with the pore platelet.

The normal formula is: 4′, 9″, a pore platelet, 12c, 7‴, 2⁗, 1p and 6s.

Discussion

The only difficulty encountered in the tabulation of this organism was the rapidity with which it exuviated its plates. Most of the individuals were found in a quiet state, short ellipsoidal in form, and without theca. The actively swimming cells were of course difficult to measure and draw. Any attempt to stop them for a moment led to cell deformation and ecdysis. This is accomplished in a very peculiar way: all the plates separate from each other, but in general, they remain surrounding the cell at a short distance, forming a regular assemblage. The plates are very delicate.

The plate pattern of this species is fundamentally different from all of those previously known (Balech, 1956; Biecheler, 1952; Dangeard, 1927; Graham, 1942; Kofoid, 1907–33; Lindemann, 1928; Schiller, 1933, 1937). The differences are in both the epi- and hypotheca. Since very little is known regarding the cingulum and sulcus of most dinoflagellates, we cannot discuss the differences concerning these regions. Nevertheless, it should be pointed out that the structure of the sulcus of this species is different from that of all sulci already studied, and no other genus is known with such a high number of girdle plates.

Other genera without epithecal intercalaries and with four apical plates are *Diplopsalis, Dolichodinium, Goniodinium, Glenodinium, Cladopyxis,* and *Ceratium.* The two latter are very different in form, bearing strong horns or arms, and with many differences in plate pattern. *Diplopsalis* as defined by Lindemann (1928) is actually an assemblage of several genera. But even on these terms, *Diplopsalis* never has more than seven precingulars, five postcingulars and it lacks the posterior intercalary. *Glenodinium,* as defined by Schiller (1933–37), is another very heterogeneous assemblage with a very large variation of plate patterns. None of its species has so many pre- and postcingular plates, and they also lack posterior intercalaries. *Dolichodinium* seems to have only six girdle plates and has six precingulars and six postcingulars instead of nine and seven. *Goniodinium* is perhaps the genus most closely related to *Fragilidium,* but it has only six precingulars and six postcingulars; instead of one posterior intercalary it has three intercalaries in the hypotheca. Until the discovery of *Fragilidium, Goniodinium* was the genus with the highest known number (nine) of cingular plates (this number, however, was not stated with certainty).

The high number of precingular, postcingular and cingular plates is sufficient to characterize this new genus. The only other genera with seven postcingulars are *Glenodiniopsis* and *Heterodinium. Pyrophacus* is the only genus with a higher number of these plates.

Fragilidium heterolobum was isolated from plankton at La Jolla (San Diego, California) on March 20, 1957.

SUMMARY

1. Two new genera and species of dinoflagellates are described. Both were originally isolated from plankton samples collected at La Jolla (San Diego, California).

2. *Scrippsiella sweeneyi* is a small species with the general tabulation of an *Orthoperidinium,* but it differs in having six cingular plates. The structure of the sulcus is also different. A great deal of variation in plate pattern was exhibited by this organism.

3. *Fragilidium heterolobum* is a medium-sized species having a tabulation that is quite different from all previously described dinoflagellates. It has a very high number of cingular plates (twelve). The generic name refers to the characteristic frequency and suddenness with which it sheds its plates.

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PHOTORECEPTION IN THE OPOSSUM SHRIMP, MYSIS RELICTA LOVÉN [1]

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Little information is available on the physiology of photoreception in the Mysidacea other than a few studies of phototaxis. Practically nothing is known of their spectral sensitivity, dark-adaptation, or lower limits of vision. The two observations that have been made on the response of the opossum shrimp, *Mysis relicta* Lovén, have shown that they swim downward when subjected to light (Dakin and Latarche, 1913) and are especially sensitive to a combination of high temperature and bright light (Larkin, 1948). The present study of the spectral sensitivity, dark-adaptation, and phototaxis of *M. relicta* was undertaken to add to our information on the physiology of photoreception.

MATERIALS AND METHODS

Mysis relicta is ideal for a study of this nature, since its large size (average length, 15.0 mm.) facilitates observation, and a laboratory population can be easily maintained. The animals used in this study were collected in Lakes Huron and Michigan.

All experiments were conducted in a cold-room under controlled light conditions and at a constant temperature of 10° C. The dark-adaptation and special sensitivity studies were carried out in an all-glass aquarium 12.0 by 8.0 by 3.0 inches, filled to 1 inch from the top. A 24-inch glass tube, with a 1-inch diameter, was used in the experiments on phototaxis.

Under conditions of continuous darkness or light, the mysids normally rested on the bottom of the tank, periodically making short excursions off the bottom. If the experimental light was turned on when the mysids were swimming upwards, they hesitated momentarily, turned, and swam rapidly to the bottom of the aquarium. This momentary hesitation was found to be a reliable indicator for the photic response.

The difficulty of observation in a dark room was met by using an infra-red viewer and infra-red light source or a large Fresnel lens to focus low-intensity red light (approximately 0.001 foot-candle) on the observer's eye. The red light was produced by a neon glow lamp and a number 2404 Corning glass filter, transmitting wave-lengths of 620 mμ and greater. It was permissible to use this light for viewing, since preliminary studies had established that *M. relicta* was almost completely insensitive to the red region of the visible spectrum in the 620-

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[1] Based on a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, University of Michigan, 1958.

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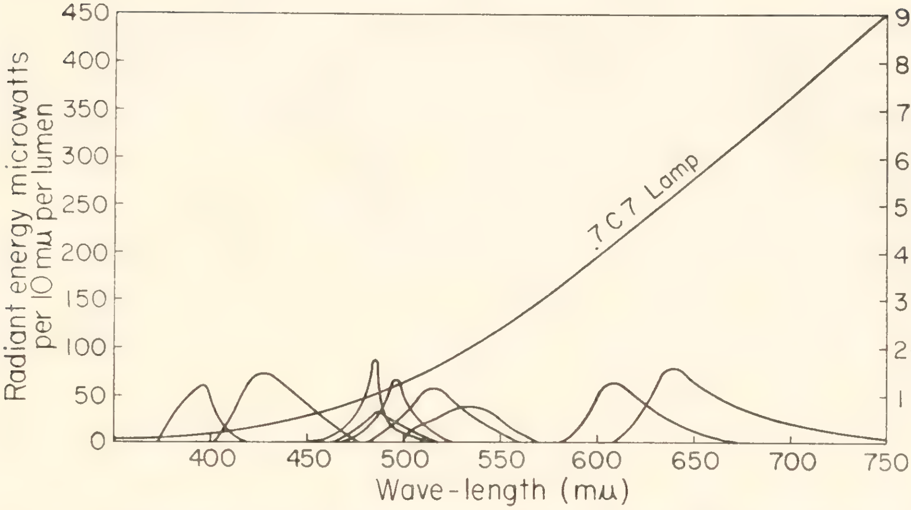

FIGURE 1. Spectral distribution of a tungsten filament lamp (color temperature 2300° K) and the spectral transmission of monochromatic filter combinations.

to 700-mμ wave-band. Mysid behavior was the same when viewed by either of the above methods.

A General Electric 7C7 tungsten filament lamp suspended 3 inches above the water surface provided the light source for the spectral sensitivity studies. This lamp has an average color temperature of approximately 2300° K and its lumen output averages 45. Although the exact distribution curve of spectral energy was not available for the lamp, a reasonably accurate curve was constructed by extrapolation from data supplied by the Nela Park Laboratory of General Electric (Fig. 1).

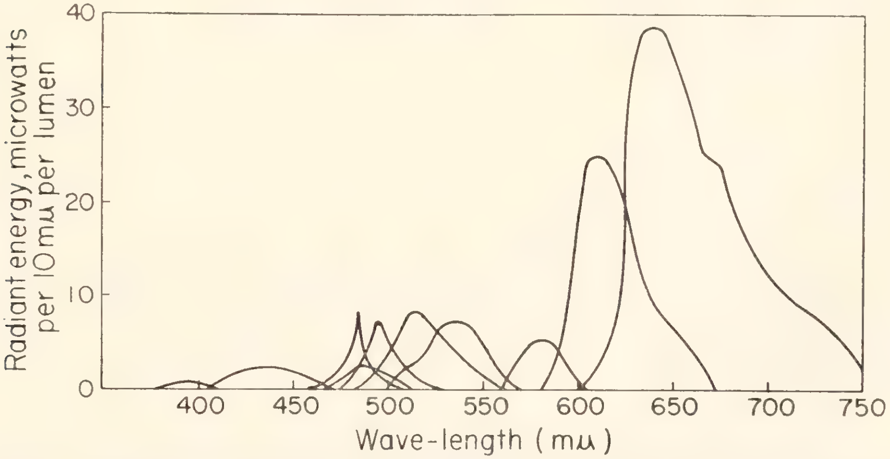

FIGURE 2. Radiant energy output from a tungsten filament lamp (color temperature 2300° K) through various monochromatic filter combinations.

Nine monochromatic-filter combinations were made with Corning glass color filters and interference filters; Figure 1 gives the spectral transmission of these filter combinations. The total energy output of a given filter combination was calculated by multiplying the energy available (in 10-mμ-wide wave-bands) from the lamp by the percentage transmission of the filter combination (Fig. 2). The percentage transmission of the various filter combinations was obtained either from data supplied by Corning (1948) or with a Beckman DU Spectrophotometer. The range of intensities for a particular wave-band was obtained by the use of evaporated-metal, neutral-density filters, having optical densities of 0.6, 0.9, and 1.0.

The reaction times of the mysids to the various intensities of radiant energy were measured by a stop watch. Sufficient time was allowed between successive tests to keep the mysids completely dark-adapted.

In the dark-adaptation experiments, the mysids were first subjected for 3 minutes to the intense light of a 1000-watt photoflood lamp. At the end of the 3-minute period all lights were turned off except the viewing light. Then the mysids were subjected to flashes of light, approximately 0.1 second in duration, of a given intensity. These flashes were spaced at 1- to 3-minute intervals. The experimental light was a 6-watt, 9-foot-candle, 115-volt, tungsten-filament lamp suspended 3 inches above the water surface. The intensity of the light was altered by interposing various numbers of evaporated-metal, neutral-density filters, each having an optical density of 1.0. The time in the dark, prior to first stimulation, was measured by stop watch.

In the experiment on the phototactic response of *M. relicta*, six individuals were placed in a 24-inch glass tube, with a 1-inch diameter, lying horizontally to eliminate any gravitational effects. The experimental light, a 7C7 lamp, was suspended 1 foot above the midpoint of the tube. After the mysids had been subjected for measured intervals to total darkness or light, one-half of the tube was shaded and the number of mysids in the unshaded half of the tube was recorded at 30-second intervals for a 5-minute period. First the right and then the left half of the tube was shaded to detect any bias in the distribution of the mysids. Control runs, with neither half being shaded, were made at frequent intervals.

Spectral Sensitivity

Earlier studies of spectral sensitivity

It has been well established that the first step in the response to light in any animal is a photochemical reaction. Hecht's (1919, 1920, 1921) work on the clam, *Mya arenaria* Verrill, contributed much toward establishing the photochemical nature of photoreception. He demonstrated that the fundamental concept of photochemistry, the Bunsen-Roscoe reciprocity law which holds that the photochemical effect is equal to the product of time and intensity, could be applied to the data of his studies.

Before a photochemical reaction can occur, light must be absorbed. The absorption spectra of the visual pigments will therefore determine the effects on the photoreceptor and in turn the response of the organism. The behavioral response of an organism to different regions of the radiant-energy spectrum has given rise to the concept of "action spectra." For example, if a visual pigment has its absorption peak at a wave-length of 500 mμ, the animal possessing this visual pigment would be most sensitive to this wave-length. This concept has led to a number of action-spectrum studies on invertebrates. Mast (1917) reported peak sensitivity to light at the following wave-lengths: 483 mμ in *Euglena, Arenicola, Trachelomonas,* and *Lumbricus;* 524 mμ in *Pandorina* and *Eudorina;* 503 mμ in *Chlamydomonas,* and blowfly larvae. Hecht (1921) established the basis for future studies through his experiments on *Mya arenaria.* This clam

TABLE I

Reaction time (seconds) of Mysis relicta to various wave-lengths of light at several intensities (Unless noted otherwise the neutral-density filters transmitted 10 per cent of the light)

Wave-length of peak transmission (mμ)	Number of neutral-density filters	Energy output of 7C7 lamp through filters (microwatts/10 mμ/lumens)	Average reaction times for 10 trials (seconds)	Standard deviation
395	0	2.20	0.77	0.18
395	*1	0.26	0.88	0.12
395	2	0.02	1.16	0.39
430	0	11.19	0.72	0.06
430	**1	2.80	0.84	0.06
430	*1	1.34	0.86	0.31
430	***2	0.34	0.92	0.16
430	****3	0.03	1.15	0.39
485	0	7.63	0.87	0.14
485	1	0.76	1.04	0.12
485	2	0.08	1.18	0.10
488	0	13.63	0.86	0.19
488	2	0.14	1.13	0.20
496	0	14.93	0.77	0.24
496	1	1.49	0.82	0.11
496	2	0.15	0.98	0.14
515	0	32.98	0.64	0.13
515	1	3.30	0.71	0.20
515	2	0.33	0.83	0.21
515	3	0.03	1.01	0.19
515	4	0.003	1.42	0.36
540	0	29.85	0.84	0.15
540	1	2.99	0.92	0.06
540	2	0.30	0.95	0.22
540	3	0.03	1.33	0.25
610	0	108.94	1.10	0.17
610	1	10.89	1.10	0.09
610	2	1.09	1.33	0.36
640	0	256.11	1.13	0.31
640	1	25.61	1.22	0.43
640	2	2.56	1.32	0.45
640	3	0.26	(No reaction)	...

* Neutral-density filter transmitting 12 per cent of the light.

** Neutral-density filter transmitting 25 per cent of the light.

*** Filters 1 and 2 combined.

**** Neutral-density filter transmitting 10 per cent of the light combined with filters 1 and 2.

had a maximum sensitivity at 490 mμ. Most insects apparently have two peaks of maximum sensitivity, 365 mμ and 492 mμ (Weiss, 1943).

Results obtained through the methods of these investigators are similar to those secured by *in vitro* studies of the spectral absorption of squid rhodopsin (Bliss, 1948). An electrical method has been employed to determine the spectral sensitivity of the eyes of *Limulus* (Graham and Hartline, 1935), a grasshopper, *Melanoplus* (Jahn, 1946), and the diving-beetle, *Dytiscus* (Jahn and Wulff, 1948). These studies demonstrated, as did the behavior method of Weiss, that the arthropod eye has a peak sensitivity in the green region of the spectrum.

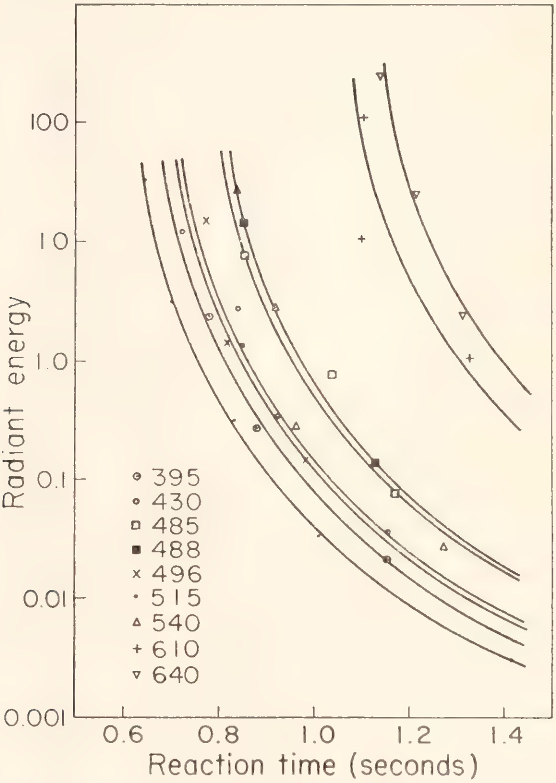

FIGURE 3. Reaction rate of *Mysis relicta* to certain wave-lengths (mμ) of light at various intensities. (Each point represents the average reaction time for 10 trials.)

The only source of information on the sensitivity of mysids to various regions of the spectrum is Hess' (1910) study of the phototactic response of a marine mysid to certain regions of the visible spectrum. Sixty-four mysids were placed in the dark and then subjected to a continuous spectrum of light. The mysids swam toward the light and aggregated in certain regions of the spectrum: 40 in the yellow-green, 19 in the blue and violet, and 5 in the red. When the position of the spectrum was altered the mysids followed the yellow-green band.

Spectral sensitivity in M. relicta

The dark-adapted mysids reacted to the different regions of the spectrum with the typical photic response but the time of reaction varied with wave-length. The mysids reacted most quickly at wave-lengths in the vicinity of 515 mμ and 395 mμ (Table I). The reaction times at wave-lengths of 610 mμ and 640 mμ were much slower (Fig. 3) despite the fact that considerably more energy was available in the red region of the spectrum than in the blue region (Fig. 2). If the mysid eye were equally sensitive to all regions of the spectrum, the faster reaction time would have been elicited by the higher energy of longer wave-lengths. The speed of response decreased progressively as the wave-length increased from 395 mμ to 488 mμ (Fig. 3). Then the trend was reversed and the mysids responded progressively faster as the wave-length increased. The reaction time at a wave-length of 496 mμ was similar to that recorded at 430 mμ and the quickest response was elicited by light with peak energy at 515 mμ. The reaction to light with wave-lengths longer than 515 mμ became progressively slower as the wave-length increased.

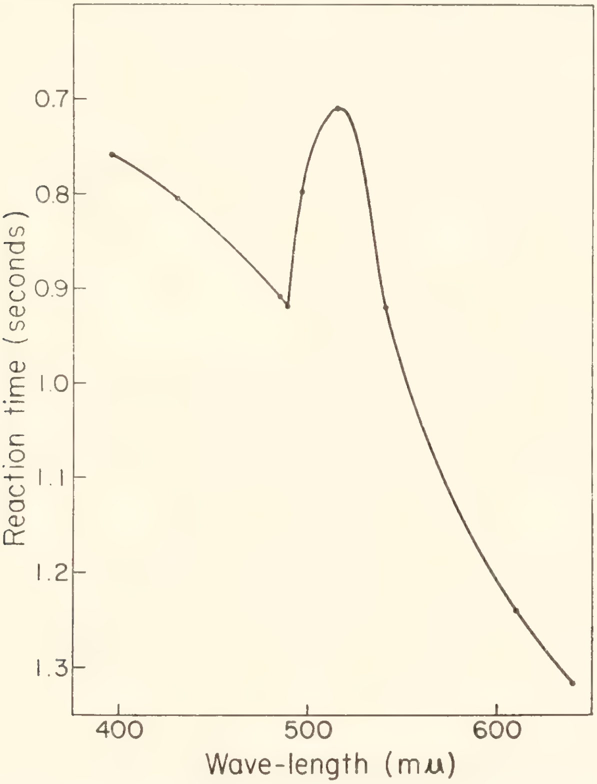

FIGURE 4. Action spectrum of the dark-adapted *Mysis relicta* eye.

The reaction times of the mysids to a wide range of intensities for a particular wave-length were determined through the use of evaporated-metal, neutral-density filters. Similar reaction times could be obtained for different wave-lengths by altering the intensity. Approximately 1000 times more radiant energy was required in the red region than in the blue-green region of the spectrum before equal reaction times could be obtained. The curves resulting from a plot of the reaction time against the radiant energy available from the 7C7 lamp and the filter combinations show that the reaction time varies inversely as the logarithm of the light intensity (Fig. 3). It is apparent that the rate of response to different wave-lengths was caused by differences in the ability of the eye to absorb energy at various wave-lengths.

The reaction times for a definite energy value of 3 as determined from the curves in Figure 3 were plotted against the transmission peaks of the monochromatic filter combinations to give an "equal-energy" curve. The resulting curve is the "action spectrum" or spectral sensitivity curve for *M. relicta* (Fig. 4). The mysids have a maximum sensitivity to light with a wave-length of approximately 515 mμ; another peak of sensitivity occurs in the violet region of the spectrum at or below 395 mμ. This suggests that the mysid eye contains at least two visual pigments.

DARK-ADAPTATION

Earlier studies of dark-adaptation

All of the photoreceptors studied by various investigators have shown an increased sensitivity to light after a period in darkness. This reaction has given rise to the concept of dark-adaptation, a process that has been studied by several different methods, including behavior. Studies on *M. relicta* have been made by a procedure similar to a behavioral method employed by Hecht (1919) to study dark-adaptation in *Mya*.

The behavior method for determining dark-adaptation has produced data that are similar to those obtained by *in vitro* resynthesis of bleached rhodopsin (Chase and Smith, 1939) and by measuring the electrical response of the eyes of *Limulus* (Hartline, 1930).

I could not find any published account of previous studies on dark-adaptation in Mysidacea.

Dark-adaptation in M. relicta

The 3-minute exposure of the mysids to the photoflood lamp evidently caused a bleaching of the visual pigment. The mysids did not respond to any light for at least 30 seconds after the lamp was turned off. Response to the experimental light after a period in darkness would indicate, therefore, that sufficient visual pigment had been resynthesized for photoreception. The light intensity necessary to elicit a response after a period in the dark was considered the "threshold intensity."

The light intensity required to produce a reaction decreased with the increase of time spent in the dark (Fig. 5). Several repetitions of this experiment gave closely similar results. The increase in sensitivity was relatively fast during the first 57 seconds, and then somewhat slower. The upper part of the curve probably lies too far to the right, since the mysids did not start to swim up until at least

30 seconds after the photoflood light was turned off; consequently, it was impossible to determine their sensitivity during this period. Possibly the steeper slope in the upper part of the curve can be attributed to a state of shock from exposure to the intense light. The initial recovery of sensitivity of the mysid eye, therefore, may progress at a rate other than indicated by the upper part of the curve in Figure 5. Light intensity values of 9×10^{-2} foot-candle and below and time in the dark gave a straight line when plotted on a logarithmic scale. These curves closely resemble that for a bimolecular reaction.

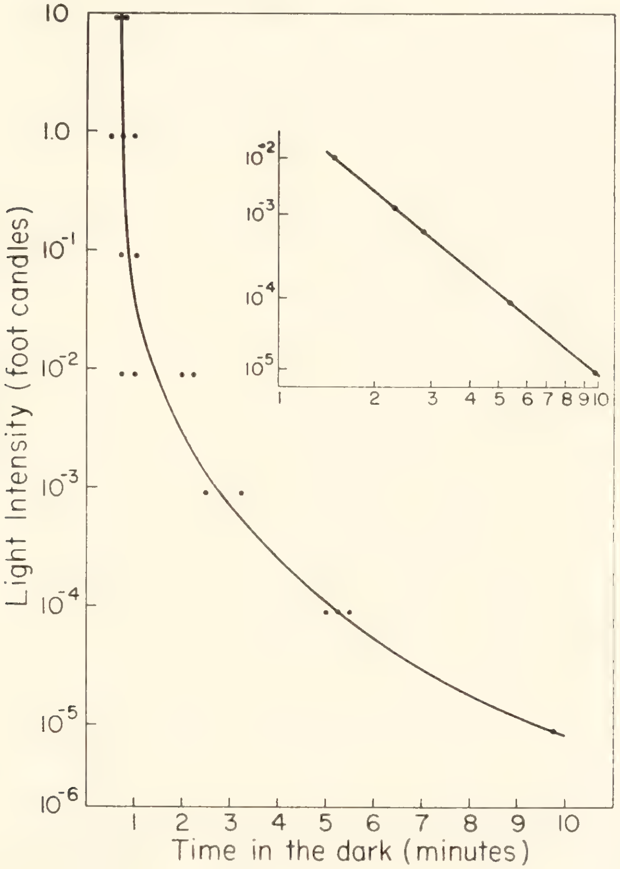

FIGURE 5. Dark-adaptation of *Mysis relicta.* Threshold intensity is plotted as ordinate against time in the dark as abscissa. Inset is the plot of log of threshold intensity against log of time in the dark.

Although a precise determination of the lowest limits of visual sensitivity could not be made, a definite response to a light intensity of 10^{-6} foot-candle was established. In all probability the mysids can detect even a much weaker light.

The question arises as to whether the stimulating flashes themselves have any effect upon the dark-adaptation of the eye. Hartline (1930) believed that if they are widely spaced in time, 3 to 5 minutes, they have little effect.

PHOTOTAXIS

Earlier studies on phototaxis

Photoreception is important for the normal orientation of mysids. Delage (1887) established that the statocysts of mysids have a balancing function, but it was subsequently demonstrated that if the statocysts are removed, the eyes make possible normal orientation in the light (von Buddenbrock, 1914). The mysids always kept their dorsal side oriented toward the light—a reaction termed the "dorsal light reflex." Additional studies on three different species of mysids have shown that orientation is governed by responses to a combination of gravitational and light stimuli (Fraenkel, 1931; Foxon, 1940). Fraenkel suggested that light stimulates the mysids to assume a position whereby a definite region of the statocyst is stimulated by gravity.

The phototactic responses of mysids are varied and complex. Keeble and Gamble (1904) reported that the phototactic sign changed from positive to negative when the mysids were moved from a white to a black background. Bauer (1908) found that mysids remained at the bottom of an aquarium when light came from above and were positively phototactic to a lateral light. Menke (1911) concluded that *Hemimysis lamornae* was positively geotactic and negatively phototactic. He also removed the statocyst and interpreted the mysids' subsequent failure to leave the bottom as a pronounced negative phototaxis. Recent research has shown that this behavior may be a "general position reflex" whereby mysids utilize the tactile sense for orientation along with the "dorsal light reflex" (Foxon, 1940). Some differences in response can be related to age. Adults of *Neomysis vulgaris* [*N. integer* Leach] are more strongly photonegative than are the young (Lucas, 1936). Mysids have been shown to be telotactic; when two light rays are crossed at right angles they react to one light source and ignore the other. Light-adapted individuals also have shown a reversal in the phototactic sign by swimming to and fro in the direction of the incident light (Fraenkel, 1931).

The observations of other workers that certain marine mysids possess a diurnal rhythm in their chromatophore system (Keeble and Gamble, 1904) suggested the possibility of a similar periodicity in the phototactic responses of *M. relicta*. Their investigations of the chemical conditions of the mysid's tissues indicated the possibility of a metabolic periodicity. The liver and muscle tissues were alkaline in the morning and became progressively acid during the day. Some of the experimental work which Menke (1911) conducted led him to postulate that *Hemimysis lamornae* was negatively phototactic during the day and positively phototactic at night. The experiment described in the next section was devised to detect such a periodicity in *M. relicta*. Although the results were not conclusive on that point, other important findings warrant inclusion of these data in this paper.

Phototaxis in M. relicta

Whenever the control runs were conducted the distribution did not differ significantly from random. The distribution did differ significantly (Chi Square test) when one-half of the tube was shaded after the mysids had been in the dark or light for a period of time. The mysids were photopositive unless they had been subjected to total darkness for 10 hours; then they became photonegative

(Table II). The photonegative condition persisted for only a short time as they became light-adapted within 6 minutes. On August 15 at 11:54 Eastern Standard Time (EST) the mysids were definitely photonegative after being in the dark for 13 hours; after exposure to the experimental light for 6 minutes they tended to be photopositive. On the same date at 21:06 EST the mysids still responded photopositively after approximately 4 hours in the dark. Similar results were secured on August 16 at 10:19 EST and August 18 at 11:55 EST, the mysids were photonegative after 13 and 14 hours in the dark. At 22:43 EST on August 16 they were photonegative after 10 hours and 14 minutes in the dark. The fact that the mysids could be photonegative either in the morning or at night indicates that the phototactic response does not have a persistent diurnal rhythm. Existence of the photonegative condition depends on the amount of time in the dark.

Table II

Phototactic response of M. relicta to a constant light intensity after periods in light and in total darkness. [Observations were made of the distribution of 6 animals at 30-second intervals for 5 minutes, except at 21:12 (5½ minutes), 11:55 (2½ minutes), 11:58 (2½ minutes). Numbers in parentheses indicate control run, i.e., entire tube unshaded. Asterisk indicates significant Chi Square value at 5 per cent level. Experiments were started at 20:54 EST on August 14, 1956, and completed at 12:06 EST on August 18, 1957]

Time (EST)	Time in dark		Time in light		Number in portion of tube		Chi Square
	Hours	Minutes	Hours	Minutes	Shaded	Unshaded	
20:54	—	—	2	54	18	42	9.6*
11:54	13	5	—	—	51	9	29.4*
12:03	—	—	—	6	25	35	1.66
13:05	—	—	1	8	9	51	29.4*
13:11	—	—	1	14	17	43	11.27*
13:37	—	21	—	—	27	33	0.6
16:38	—	—	—	—	(30)	(30)	0.0
16:51	—	—	2	56	19	41	8.06*
21:06	4	10	—	—	12	48	21.6*
21:12	—	—	—	6	28	38	1.51
21:17	—	—	—	—	(31)	(29)	0.066
10:19	12	57	—	—	36	24	2.4
10:25	—	—	—	6	43	17	11.26*
10:31			—	—	(28)	(32)	0.266
12:13	1	36	—	—	34	26	1.066
12:19	—	—	—	6	17	43	11.26*
12:24	—	—	—	—	(29)	(31)	0.066
22:43	10	14	—	—	41	19	8.06*
22:48	—	—	—	5	35	25	1.66
21:28	3	38	—	—	26	34	1.06
21:34	—	—	—	6	21	39	5.4*
21:39	—	—	—	—	(34)	(26)	1.06
11:55	14	10	—	—	20	10	3.32
11:58	—	—	—	3	14	15	0.132
12:06	—	—	—	—	(33)	(27)	0.6

Experiments revealed also that if the light intensity was increased rapidly by

1 or 2 foot-candles, the mysids swam into the shaded area, although they previously had been definitely photopositive. They adapted to this increased intensity within 2 minutes. Johnson (1938) obtained similar results with the copepod, *Acartia clausii* Giesbrecht. These copepods were placed in a glass cylinder and subjected to a step-wide change in intensity. The more rapid the change in intensity, the greater was the response. The copepods were photopositive after a period in the dark, but they swam away from the light when the intensity was increased.

Discussion

The experiments indicate that *Mysis relicta* has at least two visual pigments, one with an absorption peak at 515 mμ and another with a peak at or below 395 mμ. The maximum sensitivity at 515 mμ probably is important to the mysid for orientation in the environment, since light with a wave-band of 490 mμ to 540 mμ penetrates to considerable depth in Lake Huron (Beeton, 1958). The importance of the sensitivity to violet light (395 mμ) is not obvious, since light with a wave-band of 300 mμ to 420 mμ does not penetrate into the deep-water habitat of the mysids. Ultraviolet light has been found to cause negative phototaxis in *Daphnia pulex* (Moore, 1912), and *D. magna* (Baylor and Smith, 1957). In view of the deleterious effect of ultraviolet light, it is not surprising to find that many arthropods are sensitive to short wave-lengths and photonegative to ultraviolet light.

The mysids are able to "dark-adapt" at a relatively fast rate. This increase in sensitivity is interpreted as a function of the amount of regenerated visual purple available at each moment in the dark. The curve describing the progress of this reaction resembles that for the equation for a bimolecular reaction. The literature, however, reveals considerable disagreement as to whether the dark-adaptation data are fitted best by the equation for a bimolecular or monomolecular reaction. The reaction is probably bimolecular, but appears monomolecular when one of the reactants is in excess. Some of the discrepancies in the results obtained by different workers may possibly be explained, in part, by the data of Haig (1941). Haig's data showed that the curve of recovery after adaptation to a low intensity is decidedly different from that of recovery after adaptation to high intensity of illumination. Hartline and McDonald (1947) presented a similar series of recovery curves for single visual elements of *Limulus* after exposure to light of different intensities.

The change in the phototactic response of *M. relicta* is related to the prior presence or absence of light. Time also is involved, since the mysids were photopositive unless they had been subjected to total darkness for 10 hours or more; then they responded photonegatively.

Changes in the phototactic sign may be related to the activity of the organism. The mysids were very active in the light and not as active in the dark; possibly the increased activity results in an acidic condition in the tissues. The acidity would decrease with reduced activity. *Daphnia*, copepods, *Gammarus* (Loeb, 1918), and *Hemimysis* (Franz, 1911) can be made positively phototactic by adding a weak acid to the water. The assumption of a metabolic change due to differences in activity does not account for the rapid change from a photonegative to a photopositive condition. The possibility remains that light may control a chemical cycle in the organism. A compound, responsible for the photonegative condition, may be broken down upon exposure to light. It may require a long period of darkness for resynthesis of this compound.

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THE RESPIRATION OF UNFERTILIZED SEA URCHIN EGGS IN THE PRESENCE OF ANTISERA AGAINST FERTILIZIN[1]

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It has been shown (Tyler and Brookbank, 1956a, 1956b) that antisera against purified fertilizin, as well as antisera against homogenates of fertilized and unfertilized eggs deprived of the gelatinous coat material, increase the respiratory rate of demembranated fertilized eggs, and cause an inhibition of cleavage. The respiratory rate of blocked eggs increases to a maximum of 4 to 5 times the control rate in 20 to 40 minutes, and subsequently decreases to the rate of the controls during the next 40 minutes. Gradual cytolysis of the eggs occurs following 4–5 hours of exposure to undiluted antisera. The observed increase in the respiratory rate in the presence of immune serum is apparently without parallel in the previous literature. Haurowitz and Schwerin (1940) studied the respiration of pigeon erythrocytes in the presence of immune rabbit serum and active and inactive complement, yielding lysis and agglutination, respectively. No increase or decrease in respiratory rate was noted in either case. Sevag and Miller (1948), studying the respiration of *E. typhosa* (strain 0–901) in the presence of immune rabbit serum and active or inactive complement, found that intact, sensitized cells consumed oxygen at the same rate as the controls. However, in the presence of active guinea pig complement, the cells lysed, with an accompanying transitory increase (1.4-fold), which was followed by a decrease to one-fourth the control rate after 180 minutes. Harris (1948) measured the oxygen uptake of Salmonella cells in the presence of agglutinating rabbit serum, and found no increase or decrease in respiratory rate over a wide range of antiserum concentrations. Nowinski (1948) investigated the possibility of an effect on respiratory rate by reticulo-endothelial-immune-serum (REIS) acting on rat spleen slices, and by anti-chick brain serum acting on chick brain homogenates (1949). No effect on respiration was observed with REIS, and a slight inhibition of oxygen uptake of chick brain homogenates was noted in the presence of anti-chick brain serum. MacDonald (1949) obtained similar results with REIS using rat spleen slices in Thunberg experiments.

The purpose of the present experiments was to explore the effect of antisera against fertilizin, which is, chemically, a rather well defined substance (Tyler, 1949, 1956), on the respiratory rate of unfertilized sea urchin eggs. Unfertilized eggs, though normally respiring at a low rate, can be stimulated to respire at a much greater rate (4–5-fold increase) by parthenogenetic agents (Warburg, 1908; Keltch and Clowes, 1947), by nitrophenols (Clowes and Krahl, 1934, 1936) and other non-parthenogenetic substances, as well as by fertilization. In this connection, it is noteworthy that Perlman (1954, 1957), and Perlman and Perlman (1957) have reported that antisera against fertilizin, as well as antisera against extracts of unfertilized eggs, are capable of activating the unfertilized eggs of *Paracentrotus lividus*. The respiratory rate of eggs so treated therefore becomes of interest.

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Materials and Methods

Preparation of antigens and antisera

1) *Lytechinus variegatus* (Cedar Key, Florida). Fertilizin antigens were prepared from neutralized supernatant egg-water of acid- (pH 3.5) treated unfertilized eggs. In the case of antigen number 4, the egg-water was dialyzed against distilled water and injected without further purification. Antigen number 10 was prepared by first precipitating the fertilizin of the egg-water (derived from a second spawn of eggs) with 5/4 volumes of cold 95% ethanol (Tyler, 1949). The fertilizin precipitate was then washed thoroughly with additional volumes of cold ethanol, and vacuum-dried. The dried precipitate was dissolved in distilled water, and used for injection. Both antigens had a final agglutination titer of approximately 1/1000 on homologous sperm.

A whole sperm antigen (number 11) was prepared from washed *Lytechinus variegatus* sperm (presumably free of seminal fluid) which were diluted to a 10% suspension by volume with sea water and frozen until used.

All above antigens were stored at − 20° C. in one-ml. aliquots. After thawing for use in injections and various tests, the material remaining in each individual aliquot was discarded. This procedure avoided repeated freezing and thawing of the antigens.

Antisera against the above antigens were prepared in rabbits according to an immunization schedule described elsewhere (Tyler and Brookbank, 1956a). Following a control bleeding, totals of 400 μg N (no. 4), 200 μg N (no. 10), and 6000 μg N (no. 11) were injected into the animals over a period of three weeks. The rabbits were bled by cardiac puncture 5 days following the final injection. The antisera were recovered from the retracted clots, and dialyzed thoroughly against sea water at 10° C., and stored at − 20° C.

In addition, an antiserum against extract of washed, demembranated, fertilized eggs of *Lytechinus pictus* was available, and was used in a number of experiments. This antiserum had been previously shown to increase the respiration rate of fertilized *L. pictus* eggs (Tyler and Brookbank, 1956b).[3]

2) *Strongylocentrotus purpuratus* (Friday Harbor, Washington). A single fertilizin antigen was prepared by precipitation of the fertilizin from the egg-water of acid- (pH 3.5) treated eggs by NaOH (in the ratio of 40 ml. 1 N NaOH per liter of egg water; Tyler, 1949). The resulting precipitate was dissolved in sea water, following neutralization of the alkali, and dialyzed against distilled water.

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[3] Due to an oversight on the part of the authors, Tyler and Brookbank (1956b) contains an error on page 312, line 6. This line reads correctly if *L. pictus* is substituted for *S. purpuratus*.

TABLE I

The effect of antisera, normal sera, 0.1% sperm suspension, and hypertonic sea water on the respiration of unfertilized Lytechinus variegatus eggs

Experiment no.	Reagent employed	Aver. rate (μl./min./vessel)	Ratios of aver. rates (increased/control)
1	No. 4 normal serum	0.28	
	No. 4 normal serum	0.28	
	Anti-no. 4	0.67	2.4
	Anti-no. 4	0.67	
	Anti-*L. pictus* fertilized egg extract	0.73	2.6
	Anti-*L. pictus* fertilized egg extract	0.73	
	Buffered sea water	0.28	
	Buffered sea water	0.28	
	Buffered sea water	0.33	
2	No. 10 normal serum	0.25	
	Anti-no. 11 serum	0.31	
	Anti-no. 10 serum	1.18	4.1
	Anti-no. 10 serum	1.13	
	Buffered sea water	0.28	
3	No. 4 normal serum	0.33	
	No. 4 normal serum	0.28	
	Anti-no. 4	1.08	3.5
	Anti-no. 4	1.03	
	0.1% sperm suspension	1.07	3.5
	Buffered sea water	0.33	
	Buffered sea water	0.28	
	Buffered sea water	0.22	
4	No. 5 normal serum	0.30	
	No. 5 normal serum	0.22	
	Anti-*L. pictus* fertilized egg extract	0.92	3.4
	Anti-*L. pictus* fertilized egg extract	0.84	
	Buffered sea water	0.27	
5*	No. 11 normal serum	0.14	
	Anti-no. 10	0.95	6.7
	Anti-*S. purpuratus* fertilizin (three antisera)	0.37	2.6
		0.33	2.3
		0.33	2.3
	Buffered sea water	0.14	
6	0.1% sperm suspension	0.83	3.2
	0.1% sperm suspension	0.80	
	Hypertonic sea water	0.84	3.3
	Hypertonic sea water	0.84	
	Buffered sea water	0.25	
7	Anti-no. 10 undiluted	0.32	2.5
	Anti-no. 10 1:1 dilution	0.26	2.0
	Anti-no. 10 1:2 dilution	0.24	1.8
	Anti-no. 10 1:4 dilution	0.20	1.5
	No. 11 normal serum	0.16	
	Buffered sea water	0.10	
	Buffered sea water	0.12	

* In experiment 5, one ml. of 20% egg suspension was used with vessels of 7-ml. capacity, as opposed to 2.3 cc. of egg suspension with vessels of 15-ml. capacity in the other experiments. Five-tenths ml. of antiserum or normal serum was used throughout all experiments. The conditions in experiment 5 duplicate those which obtained during experiments with eggs of *S. purpuratus*.

After dialysis, the fertilizin was precipitated with 5/4 volumes of cold ethanol, washed, vacuum-dried, redissolved in distilled water, and used for injection into each of three rabbits. This antigen had a sperm agglutination titer of approximately 1/500.

Following a control bleeding, the rabbits were injected according to a previously described schedule (Tyler and Brookbank, 1956a) for a period of 2½ weeks, each receiving a total of 250 μg N. The animals were bled by cardiac puncture 4 days following the last injection.

Manometric methods

1) *Lytechinus variegatus.* The effect of the antisera on the respiration of unfertilized eggs, deprived of all soluble fertilizin by acid (pH 3.5) treatment, was followed, at 20° C., in a standard Warburg apparatus using vessels of approximately 15 ml. total capacity ($k_{O_2} = 1.4$–1.5 μl./mm.). The eggs used in these experiments were 90–100% fertilizable following the acid treatment. The settled eggs were diluted to a 20% suspension, on the basis of settled volume, with buffered sea water (0.01 *M* glycyl-glycine, pH 8.0). The suitability of glycyl-glycine as a sea water buffer, and as a medium for the eggs, has been established by Tyler and Horowitz (1937). The main chamber of the vessel contained 2.3 ml. of egg suspension, the center well 0.2 ml. of 10% NaOH, and the side arm 0.5 ml. of the test solution (antiserum, normal serum, hypertonic sea water, or sperm suspension). After the respiratory rate of the eggs in buffered sea water had been established (usually after 40 minutes), the side arms were tipped and the rate of respiration in the test solution was determined. At the end of each experiment, the eggs were examined microscopically.

2) *Strongylocentrotus purpuratus.* During the two preliminary experiments reported in Table II, the procedure followed was similar to that described above, with the following exceptions: (1) The temperature employed was 18° C., the maximum tolerated by these eggs. (2) One ml. of a 20% suspension, in buffered sea water, was exposed to 0.5 ml. of serum, added from the side arm of vessels of ca. 7-ml. total capacity ($k_{O_2} = 0.7$–0.8 μl./mm.). Thus, the ratio of ml. of serum to the final volume in the vessel was increased, in these experiments, from 0.18 to 0.33 ml. serum/ml. (3) In experiment 2, Table II, the egg suspension was exposed to 1 mg% trypsin solution (crystalline, lyophyllized trypsin—Worthington Biochemical Corp., Freehold, N. J.) for 10 minutes prior to suspension in buffered sea water. This treatment prevented the elevation of the fertilization membrane of test eggs inseminated in sea water following washing. The eggs used in both experiments were 95–100% fertilizable after acid treatment. Trypsin treatment reduced this figure to 30% in experiment 2, using the same amount and concentration of sperm suspension for each test insemination.

RESULTS

As can be seen in Table I, all antisera against fertilizin increased the respiratory rate of unfertilized Lytechinus eggs, including those antisera directed against *S. pupuratus* fertilizin. The increase noted in heterologous antisera was somewhat less than that observed using homologous antisera. Normal sera and antiserum against sperm effected no measureable increase in respiratory rate compared with the rate observed in buffered sea water. The increased respiration in the presence of antisera against fertilizin reached a maximum at 20–40 minutes following the addition of the serum from the side arm. Following this, the rate declined toward the control level, reaching this point in approximately 40 minutes. The rates shown in Table I represent average rates over the entire period of exposure (80 minutes) of the cells to the sera. Maximum rates ranged from 4 to 5 times control rates. Serial dilution of the antiserum (in this case, the antiserum directed against antigen 10 was employed) progressively lowers the value of the maximum observed rate, as indicated in experiment 7, Table I. In experiment 5, dealing with the effects of heterologous antisera directed against the *S. purpuratus* fertilizin antigen, a higher proportion of antiserum relative to the amount of egg suspension was employed in order to duplicate conditions existing during experiments with *S. purpuratus* eggs. The maximum rate observed with heterologous antisera was about three times the control rate. The high (ca. 7 times the control rate) rate observed with the homologous antiserum in this experiment presumably reflects the higher concentration of antibody employed.

Table II

The effect of antisera against fertilizin on the respiratory rate of unfertilized Strongylocentrotus purpuratus eggs

	Aver. rate (μl./min./vessel)	
Experiment No. 1		
Antiserum from rabbit:		
d.	0.15	
e.	0.15	0.15 aver.
f.	0.15	
Pre-injection control serum:		
d.	0.14	
e.	0.14	0.14 aver.
f.	0.14	
Experiment no. 2 (trypsinized eggs)		
Antiserum from rabbit:		
d.	0.19	
e.	0.21	0.20 aver.
f.	0.20	
Pre-injection control serum:		
d.	0.16	
e.	0.17	0.16 aver.
f.	0.16	
Aver. rate, buffered sea water (12 determinations)	0.17 (Range—0.15–0.18)	

As controls, some aliquots of eggs were exposed to sperm suspensions (0.1%) or to hypertonic medium (1 *M* final concentration with respect to NaCl) sea water, added from the side arm of the vessel. The increased respiration observed as a consequence of fertilization or treatment with hypertonic sea water approximates the increases obtained using homologous antisera against fertilizin (Table I). Increased respiration following exposure to hypertonic medium was first reported by Warburg (1908), and confirmed by Keltch and Clowes (1947). The antiserum against extract of fertilized *Lytechinus pictus* eggs, washed and demembranated prior to homogenization, also proved effective in increasing the rate of respiration of *L. variegatus* eggs (results included in Table I).

Two preliminary experiments using unfertilized *S. purpuratus* eggs yielded less conclusive results. Slight increases in respiratory rate of questionable significance occurred after the addition of homologous antiserum against fertilizin. The increases in rate usually appeared within 30 minutes following addition of the sera, and lasted for 15–20 minutes, after which time the rates returned to the control level. Table II presents average rates during the time (60 minutes) the eggs were exposed to the sera. In experiment 2, the eggs were trypsinized as described above, on the assumption that a barrier exists at the egg surface which prevents combination of antibodies with the necessary sites on the egg. This treatment did not appreciably alter the results obtained during experiment 1. That the antisera against *S. purpuratus* fertilizin contained antibodies capable of increasing respiratory rate can be seen by the ability of these antisera to increase the respiration of *L. variegatus* eggs (Table I). In addition, these antisera were capable of blocking the first cleavage of demembranated fertilized *S. purpuratus* eggs, indicating the presence of antibodies against fertilizin (Tyler and Brookbank, 1956a, 1956b). Furthermore, positive ring precipitin tests were obtained with the homologous antigen. The problem of the failure of these antisera to cause an increase in the rate of respiration of the unfertilized eggs of *S. purpuratus* comparable to that observed using Lytechinus eggs remains unsolved at this writing. Since only two experiments are available, it may well be that future work will resolve the apparent difference between these two species.

Microscopic examination of Lytechinus eggs following these experiments revealed no morphological evidence of activation, excepting, of course, the cases in which the eggs had been inseminated. Samples of Lytechinus eggs treated with immune serum or hypertonic sea water in the manometer vessels were transferred to fresh sea water and observed periodically for 3 to 4 hours. No indications of membrane elevation or cleavage were seen in these eggs, though control eggs inseminated in the manometer vessels elevated fertilization membranes while in the vessels, and developed normally following transfer to fresh sea water. In some instances, Lytechinus eggs exposed to antisera against fertilizin were agglutinated (experiments 2, 3) and some darkening of the cortical region was noted. In most instances, the eggs tended to cytolyze in the antisera against fertilizin after 3–4 hours exposure.

DISCUSSION

The above results indicate that antisera against fertilizin are capable of temporarily elevating the respiratory rate of unfertilized as well as fertilized (Tyler and Brookbank, 1956b) Lytechinus eggs. The time course followed by the inincrease to the maximum rate and the subsequent return to the control rate is similar in both cases. Judging from the increased maximum rate in the presence of a relatively greater amount of antiserum (Table I, experiment 5), it is probably safe to assume that the rate obtained in a given experiment is a function of antibody concentration, provided the total volume and the number of eggs present remain constant. This is also indicated by experiment 7 (Table I), in which serial dilutions of antiserum against antigen number 10 were tested.

In comparing the results presented in this report with results obtained previously by others, it is apparent that increased respiratory rate in the presence of specific immune serum is seldom encountered, even over a rather wide range of biological material. The report by Sevag and Miller (1948) represents the only instance encountered by this author in which a temporary increase was observed. The increased respiratory rate was found only upon lysis of the *E. typhosa* cells in the presence of active complement, and was not observed when the cells were agglutinated. Complement was not added to the sera employed in the present study, nor were the sera heated to inactivate complement. Thus, the role of complement in the system causing the increased respiration of Lytechinus eggs is not known, though heating antisera against fertilizin to 56° C. for 30 minutes to inactivate C′1 and C′2 does not alter the cleavage blocking property of these antisera (Tyler and Brookbank, 1956a). The unfertilized eggs do not cytolyze during the period of measurement of respiratory rate, and remain intact for 3 to 4 hours following removal from the manometer vessels. After this time, a gradual cytolysis becomes evident. The increased oxygen consumption does not appear, therefore, to be associated with visible cytolytic changes in the egg, since the maximum respiratory rate in the presence of antisera against fertilizin is observed 20–40 minutes following the addition of the antisera from the side arm.

In considering the reports of Perlman (1954, 1957) and Perlman and Perlman (1957) on the parthenogenetic properties of antisera against extracts of unfertilized eggs and against fertilizin, one might be tempted to consider the increased rate of respiration of unfertilized eggs in the presence of antisera against fertilizin, or in hypertonic media, to be indicative of activation. This conclusion does not seem warranted by the data presented in this report. No morphological evidence of activation was encountered during the experiments, and one might more properly consider the increased respiration of eggs so treated to be analogous to the increases obtained in the presence of nitro-phenols (Clowes and Krahl, 1934, 1936; Krahl, 1950), methylene blue (Ballentine, 1940) and other substances which are not considered parthenogenetic. With regard to the failure to observe activation of eggs exposed to hypertonic sea water, it should be recalled that the time of exposure of unfertilized eggs to the proper hyper- or hypotonic medium is critical (Harvey, 1940). Therefore, failure to observe morphological signs of activation under the conditions prevailing in the manometer vessels is not surprising. In addition, a wide range of hyper- or hypotonic media are capable of eliciting increased respiration of the unfertilized eggs without effecting activation. The extent of the increases obtained depends on the degree of hyper- or hypotonicity employed, in much the same way as the extent of increases obtained with antisera against fertilizin depends on the amount of antibody present (Table III).

In conclusion, it seems appropriate to consider the purity of the fertilizin antigens used for injection. Special precaution was taken in the preparation of the *S. purpuratus* fertilizin antigen, and antigen number 10 (*L. variegatus* fertilizin), to insure minimum contamination with material from the eggs. These antigens were purified according to methods designed to yield electrophoretically homogeneous fertilizin, and were injected in exceedingly small amounts. The initial removal of fertilizin from the eggs was carried out at pH 3.5. Eggs treated at this pH for 2–3 minutes and returned to pH 8 develop normally, ruling against damage to the eggs by this degree of acidity. Antigen number 4 (*L. variegatus* fertilizin) was obtained in the same manner as number 10, except that the step involving precipitation of the antigen with ethanol was omitted. Results obtained using antisera against antigen number 10 paralleled those obtained using antisera against antigen number 4 completely. Apparently antisera against purified fertilizin are capable of temporarily raising the respiratory rate of the unfertilized egg. Results obtained using antiserum against extract of fertilized eggs parallel those obtained using antisera against fertilizin, since this antiserum also increased the rate of respiration of the unfertilized eggs. Since antibodies most probably cause their effects through combination with fertilizin at the egg surface, this last mentioned result seems to indicate the presence of fertilizin haptens in the fertilized-egg antigen. The most probable location of these fertilizin haptens is the hyaline layer (ectoplasmic layer) of the fertilized egg, as proposed by Tyler and Brookbank (1956a).

TABLE III

The effect of varying degrees of hyper- and hypotonicity on the respiration of unfertilized Lytechinus variegatus eggs. Conditions the same as those prevailing for the experiments in Table I. (Hypertonicity in terms of excess NaCl, hypotoncity in terms of added distilled water)

Tonicity	Aver. rate µl./min./vessel
Experiment 1—hypertonic media	
2.9 × sea water	0.97
1.85 × sea water	0.55
Sea water	0.20
Experiment 2—hypotonic media	
0.84 × sea water	0.50
0.75 × sea water	0.58
0.66 × sea water	0.67
Sea water	0.28

SUMMARY

1. Homologous antisera against purified fertilizin, and against extract of fertilized eggs (of *Lytechinus pictus*) have been shown to temporarily increase the respiratory rate of the unfertilized eggs of *L. variegatus*. Parallel experiments employing antisera against fertilizin of *Strongylocentrotus purpuratus* and unfertilized *S. purpuratus* eggs yielded essentially negative results. Further experimentation is necessary before this apparent difference between the responses of the eggs of these two species to antisera against fertilizin can be resolved.

2. Antisera against fertilizin of *S. purpuratus* were effective in increasing the respiratory rate of unfertilized *L. variegatus* eggs, indicating the presence of antibody capable of effecting increased respiration.

3. Normal sera and antiserum against sperm were without measurable effect on the respiratory rate of *L. variegatus* eggs.

4. None of the eggs treated with antisera against fertilizin showed morphological evidence of activation.

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A BICOLORED GYNANDROMORPH OF THE LOBSTER, HOMARUS AMERICANUS

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Lobsters with sharply defined, bilateral color differentiation have been described by several authors. Herrick (1896) mentioned the following variations of this pattern in both the American and the European lobster: half normal color and half light sky blue; half normal and half pale red; half greenish black and half light orange; half blue and half white; and half light yellow and half bright red. Schaanning (1929) gave a color figure of a European lobster that was light red and dark blue. Templeman (1948) added records of two more bicolored American lobsters, one normal and red, the other whitish red and purplish blue. Such color variants have occasionally been referred to as gynandromorphs or hermaphrodites, but there is no evidence that any of the previously recorded bicolored specimens were also bisexual.

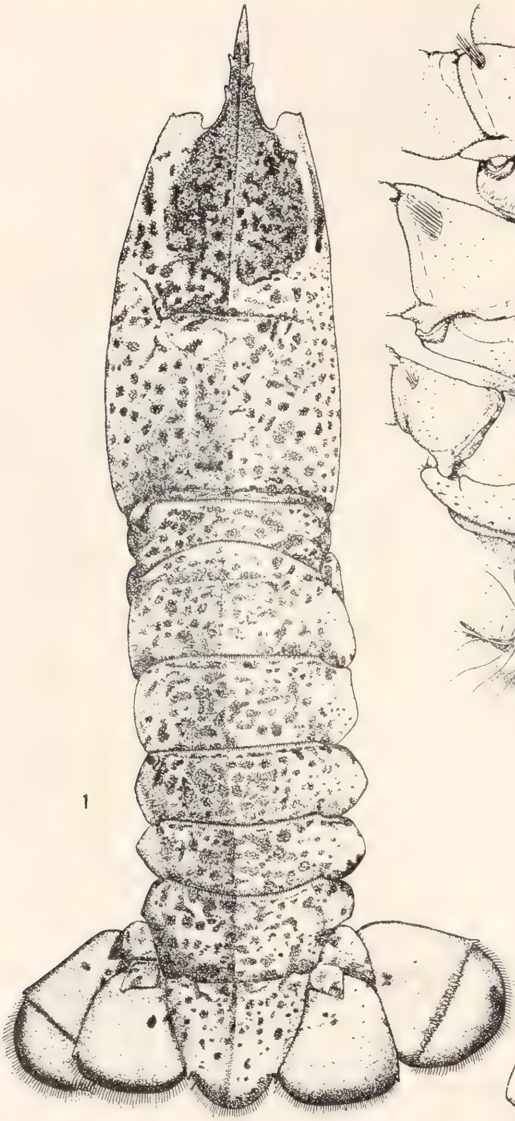
Only two cases of possibly complete hermaphroditism have been recorded heretofore for *Homarus*. Nicholls (1730) described and figured a specimen of the European lobster, *H. gammarus,* received from Newgate-Market, London, that displayed all of the external and internal female characters on the right side and all of the male structures on the left. Halkett (1919) collected a specimen of *H. americanus* at Bay View, Pictou County, Nova Scotia, November 1917 "which was absolutely male on the left side and absolutely female on the right side"; this specimen was sent to Queen's University, Kingston, Ontario, but apparently no complete description of it has been published. Gordon (1957) described a specimen of *H. gammarus* from off Seahouses, Northumberland, that had all of the characters of a perfect gynandromorph—female on the right side, male on the left —except that there was no male opening on the left fifth pereiopod but, instead, an imperforate indication of an opening on the coxa of the left third pereiopod; this specimen was not dissected, but Dr. Gordon suggests that "it probably has a normal ovary on the right side and part ovary, part testis on the left side—or a testis with ova in the anterior position." Herrmann (1890) discovered that eggs are occasionally developed during spermatogenesis in the lobster testis but he gave no indication that this was associated with any unusual external characteristics. Finally, Ridewood (1909) recorded an ovigerous specimen of *H. gammarus,* presumably from off the Orkney Islands, that had genital openings on the third right pereiopod and on the fourth and fifth left pereiopods, but dissection disclosed only a normal paired ovary with apparently three oviducts, two of them on the left side leading to the abnormally placed openings.

The specimen described below (U. S. Nat. Mus. Cat. No. 102241) seems to be the first lobster to be recorded in which a color anomaly was associated with gynandromorphism. The specimen was alive when presented to the Fish and Wildlife Service at Woods Hole, Massachusetts, during the summer of 1954 by a dealer operating between Boston and Cape Cod. Its place of origin is unfortunately unknown; it probably came from Massachusetts Bay but it could have been shipped from New Hampshire, Maine, or even Canada. It died while being photographed by John P. Wise, who offered it to the junior author for description. After remaining in a freezer for about six months, it was transferred to formalin for dissection; the dissected portion is stored in ethyl alcohol, and the carapace, abdominal tergites, tail fan, and chelipeds have been dried.

The specimen was about 10 inches (25 cm.) long from the tip of the rostrum to the end of the telson. The carapace measures 86 mm. from the eye socket to the hind margin. To the left of the midline of the animal, as well as on most of the gastric and hepatic regions on the right side, the ground color was orange, mottled and spotted with dark, greenish brown. The right side, posterior to the mesogastric and hepatic regions, was similarly spotted and mottled but in shades of blue over a lighter blue ground color, reminiscent of the colors of willow-pattern china. The color pattern is indicated in Figure 1. The spotting and mottling pattern seems to be continuous across the midline; only the color is different. The color transparency made from the living animal suggests that blue pigment was largely lacking on the left side, and red, yellow, and possibly black pigments were missing on the right side, although there is a greenish cast to the dark mottling on the left and a pinkish tinge in some of the light blue areas on the right.

The left, crusher cheliped was colored like the left side of the body for the most part, but the color photograph suggests that there were blue patches at the outer, distal end of the merus, on the dorsal surface of the carpus, on the thick portion of the hand, near the base of the fixed finger, and on the base of the dactyl. The spines on this cheliped were bright red. The right, cutting cheliped was blue, with a tinge of brown near the base of the fixed finger, and the spines were almost pure white. The other pereiopods on the left side were orange with greenish brown shadings similar to those on the adjacent portion of the carapace. Those on the right side were very light, pinkish blue with darker blue shadings. The left uropods were orange with greenish brown margins, and those on the right, pale, pinkish blue with dark blue marginal bands. The fringe of setae on the left uropods and on the telson to the left of the midline were reddish orange, those on the right side, yellowish orange.

As in the three previously described gynandromorph lobsters, the specimen displayed female characters on the right side and male structures on the left. The body was skewed to the right anteriorly and to the left posteriorly as shown in Figure 1. This was almost certainly a result of the differential growth of male and female lobsters. Templeman (1944) stated: "At all commercial sizes the relative length of the carapace is less in the female than in the male. It remains constant when the lobsters are small, but shows a definite and progressive increase for those localities for which large lobsters were available for measurement. . . . The ratio of the greatest width of the carapace to total length is in the smaller relatively immature lobsters approximately the same for both males and females, while in the larger animals it increases with size and more so in the male than in the female." The skewing of the axis and the more swollen left (male) side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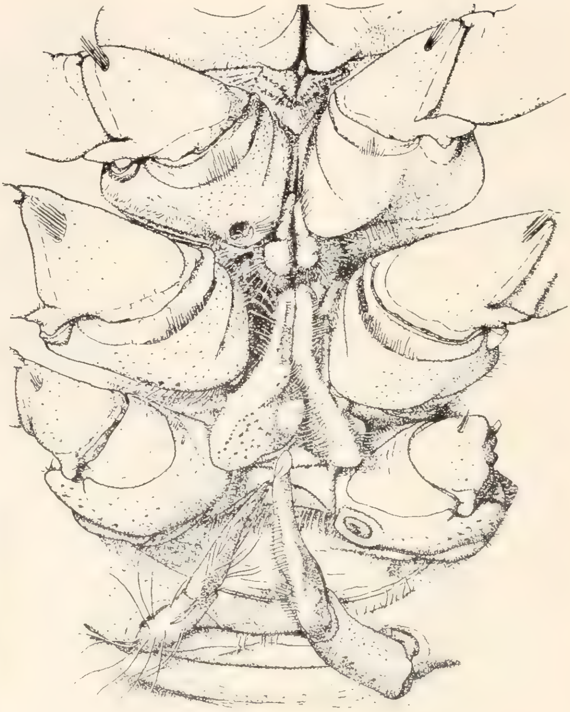
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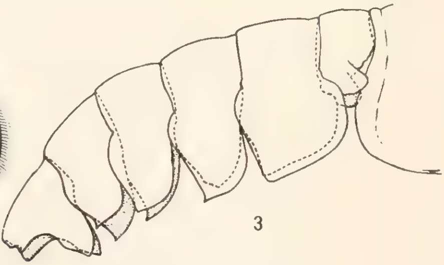
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FIGURE 1. Dorsal surface of body of gynandromorph of *Homarus americanus* showing asymmetry and color pattern. Drawn from color transparency of living animal.

FIGURE 2. Ventral surface showing opening of oviduct on coxa of third right pereiopod and of vas deferens on fifth left pereiopod, asymmetrical thelycum, and characteristic female and male first pleopods.

FIGURE 3. Abdominal somites viewed from the right (female) side. The left (male) pleura are shown as if viewed from the inside.

of the carapace are therefore readily understandable. The reverse skewing of the abdomen probably also resulted from this differential growth, accentuated by the proportionately larger abdominal pleura in the female, as shown in Figure 3. The greater apparent length of the left (male) pleura of the fourth, fifth, and sixth somites in this figure is misleading and is caused by the fact that the left (male) pleura curve downward nearly vertically, whereas those on the right (female) side extend obliquely outward, as shown in Figure 1. The distortion is less striking than in Gordon's larger (11-inch) specimen of *H. gammarus* and in Nicholls' specimen of the same species, the size of which was not given. This might be expected from Templeman's (1944) findings that the differential growth of males and females becomes progressively more marked with age.

The most noticeable disparity in our specimen is found on the ventral surface. As shown in Figure 2, there is a female opening on the coxa of the right third pereiopod and a male opening on the left fifth pereiopod, which is represented only by the coxa and basis. The thelycum is distinctly asymmetrical, the right (female) part being broad and nearly bare and the left (male) part narrower and provided with long hairs. Neither element of this structure corresponds exactly with its form in normal males and females, but the similarity to the conditions in the appropriate sexes is more than superficial. Even the median plate extending forward from the last thoracic sternite is modified as would be expected: the right portion is longer and acute, like half of a typical female plate, and the left portion is shorter and rounded as in the male.

The first right pleopod is typically female, flexible and long-haired, while the left one is a rigid male intromittent organ. There is a well-developed appendix masculina on the endopod of the left second pleopod, but none on the right member of this pair. The second, third, fourth, and fifth pleopods on the left (male) side are 37.5, 35.3, 33.5, and 29.0 mm. long, respectively, from the basal articulation to the end of the endopod. Those on the right (female) side have corresponding lengths of 37.2, 38.5, 38.0, and 33.3 mm. These figures agree remarkably well with the proportionate lengths of these appendages in normal males and females, as determined by Templeman (1944): "In the male the swimmerets (including protopodite and endopodite) on the second abdominal segment are the longest and in the female those on the third and fourth are the longest and approximately equal. The second swimmerets are not greatly different in length in males and females. . . ." The sternal spines of the gynandromorph are about 2.5 mm. long on the second, third, and fourth abdominal somites and about 2.0 mm. long on the fifth somite. Data given by Templeman (1944) for New Brunswick specimens indicate that the average length of the spine on the second somite in specimens of comparable size is about 3.75 mm. in males and 0.5 mm. in females, and the spine on the fifth somite is about 2.5 mm. long in males and 0.5 mm. in females. The spines in the gynandromorph therefore seem to be intermediate in size, perhaps more nearly approaching the male than the female condition.

Careful removal of the carapace and abdominal tergites of the specimen disclosed a well-developed ovary filled with maturing eggs on the right side and a normal testis on the left, as shown in Figure 4. An oviduct led from beneath the ovary to the opening on the right third pereiopod and a typical vas deferens connected the testis with the left fifth pereiopod. A few of the eggs in the ovary appeared slightly discolored. Herrick (1911) stated that the presence of orange flecks in the ovary, representing degenerating eggs that were not shed, is conclusive evidence that a lobster has already spawned at least once, but the spots in our specimen were not sufficiently distinct to permit an unequivocal determination that spawning had occurred. As can be seen in Figure 4, a lobe of the ovary, probably representing the connection between the two halves of the organ in a normal female, was found just anterior to the heart. At this point the testis was interrupted and the two parts of it were continuous with the intermediate portion of the ovary. It appeared that the two portions of the testis were differentiated parts of a single organ. There is no doubt that the testis was functional, for sections showed active spermatogenesis. Normal, fully formed spermatozoa were extracted from the vas deferens.

It is, of course, impossible to determine whether this specimen could have functioned reproductively as either or both a male and a female lobster. Viable spermatozoa and eggs were probably produced, but the unpaired intromittent organ and the deformed thelycum might have prevented successful copulation with normal males and females. The specimen must have been nearly or quite mature. As mentioned above, we were unable to determine whether or not it had spawned. If it was caught off the Massachusetts coast, it was probably six or seven years old according to Herrick (1911). Herrick (1896) also maintained that "very few lobsters under 9 inches in length have external eggs, while only few have attained the length of 10½ inches without having them." Templeman and Tibbo (1945) concluded from the examination of New Brunswick specimens that the length of males at sexual maturity is at least 5 cm. less than that of females. One can hardly assume, however, that the size of a gynandromorph is directly comparable with that of a normal individual of either sex. Female lobsters probably grow more slowly than males (Herrick, 1911), and one might therefore expect the present specimen to be smaller than a normal male and larger than a normal female of the same age, but the growth rates of abnormally bisexual crustaceans may be complicated by hormonal or other factors.

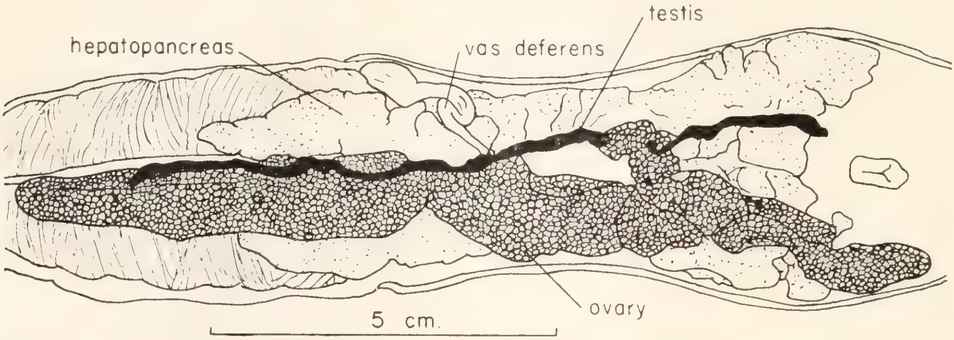

FIGURE 4. Dissection of lobster gynandromorph after removal of heart and stomach showing well-developed ovary on right side and testis and vas deferens on left.

We wish to thank John P. Wise for calling our attention to this unusual specimen. We also wish to acknowledge the assistance of members of the staff of the

Division of Marine Invertebrates, U. S. National Museum, during the preparation of the paper. Special thanks are due Charles E. Cutress of that staff for the histological preparation and study of the reproductive organs of the specimen.

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RE-EXAMINATION OF AN INHIBITOR OF REGENERATION IN TUBULARIA

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In the marine hydroid *Tubularia* the presence of hydranth structures has been thought to prevent the development of new hydranths in nearby stem tissue. Two preparations have been made from adult hydranths which inhibited the regeneration of new hydranths on isolated stem segments. One of these (*inhibitor water,* Rose and Rose, 1941) was made by agitating adult hydranths in aerated sea water for from 12 to 24 hours, while the other (*hydranth extract,* Tardent, 1955) was found in the supernatants of homogenates of adult hydranths. These inhibitors of regeneration were specific to hydranth tissue in that they were not obtained when stems were treated in the same manner. They have been compared (Tweedell, 1958) and found to differ in a number of properties. The regeneration-inhibiting substances in inhibitor water have been considered by a number of authors to represent the substances normally responsible for physiological dominance in *Tubularia,* and inhibitor water has been employed by Steinberg (1954) in an experiment to indicate the mechanism of physiological dominance.

In the present investigation it was found that active inhibitor water could not be prepared in the absence of bacterial growth, and as a consequence a re-examination of this inhibitor was undertaken.

MATERIALS AND METHODS

Freshly-collected *Tubularia crocea,* provided by the Supply Department of the Marine Biological Laboratory, was used in all experiments. Sea water was filtered through paper shortly before use.

In preparing inhibitor water, an attempt was made to use methods comparable to those used by previous workers (*cf.* Tweedell, 1958). Populations of adult hydranths with 5 mm. of stem attached were isolated and washed thoroughly, and then aerated in sea water for 24 hours at 17–22° C. After aeration, the hydranths and debris were removed by filtration and the preparation was tested for its effect on regeneration.

The bacterial population was estimated subjectively in early experiments by turbidity and microscopical examination, and in later experiments was determined using a Petroff-Hauser bacteria-counting slide. The bacterial population of filtered sea water was found to be approximately 10^5 per ml., which is too low for accurate estimation with a counting slide. It was assumed that no bacterial proliferation had occurred during the preparation of any given solution if the bacterial population did not exceed this order of magnitude. It should be cautioned that if mature male hydranths are used to prepare inhibitor water, turbidity may in part result from the release of large numbers of sperm into the water.

When it was desired to remove bacteria, the preparations were filtered through an HA millipore filter (Millipore Filter Corp., Watertown, Mass.) or centrifuged for 5 minutes at 30,000 g. Bacterial growth was prevented by the addition of antibiotics. Penicillin and streptomycin were used at 100–125 μg./ml.; sulfadiazine was used at about 0.001 per cent (or saturation in sea water). At these concentrations, and in the cases of penicillin and streptomycin even at four-fold higher concentrations, the antibiotics did not have any significant effect on the rate or course of regeneration.

The solutions to be described were tested immediately after preparation for their effect on the regeneration of freshly-cut, 7-mm. stem segments. Virtually all of the stems in control groups regenerated, although as is usual with *Tubularia* there was a considerable variation in the rate, even within a single group. A preparation was considered to have inhibited regeneration if, during the time required for the complete regeneration of the controls (emergence), all or a significant fraction of the experimental group either disintegrated or healed but did not begin regeneration. Stems in inhibitor water which regenerated were usually but not always retarded.

RESULTS

Populations of 1.5–2 hydranths per ml. aerated in sea water regularly produced an inhibitor water which completely prevented regeneration. Occasional batches of inhibitor water prepared at these or lower hydranth densities were inactive, while populations of more than two hydranths per ml. usually gave a preparation which caused disintegration of the stem tissue. However, considerable variability was found in the activity of preparations made at the same hydranth densities and under the same conditions (temperature, time, etc.), suggesting that some factor other than those controlled was involved.

A number of observations suggested that the activity of inhibitor water was due to bacterial growth. Preparations became quite turbid during the course of aeration, and the condition of the hydranths deteriorated rapidly. The solution developed a putrid odor. Hydranths killed by exposure to 30° C. for 15 minutes rapidly disintegrated, but nevertheless produced active inhibitor water. When active preparations were examined microscopically, a large, heterogeneous population of bacteria was found. Removal of these bacteria often resulted in a reduction but never in an elimination of the inhibitory activity of a preparation.

An estimate of the amount of bacterial growth which occurs in inhibitor water preparations was obtained by preparing a series of 5 inhibitor waters at a density of 1.5 hydranths per ml. and making counts with a bacteria counting slide at the beginning and end of aeration. In this series, the bacterial density increased during aeration from about 3×10^5 bacteria per ml. to about 10^8 bacteria per ml. Bacteria were removed by centrifugation and each preparation tested for its effect on the regeneration of 10 stems. The results are given in Table I. The increase in bacterial number represents a minimum of 9 generations of bacterial growth. It should be noted, however, that counts made at the beginning of aeration do not include bacteria which are present in the hydranths and are released into the water during aeration as the hydranths disintegrate.

In order to determine whether or not hydranths could produce active inhibitor water in the absence of bacterial growth, hydranths were agitated in sea water containing antibiotics at concentrations sufficient to maintain bacteriostasis. The results of three of the experiments with penicillin and streptomycin are given in Table II. In the first two experiments shown (A and B), the amount of bacterial growth was estimated subjectively. No detectable bacterial growth occurred in any of the preparations in experiment A, and in spite of the fact that the hydranth density was more than twice that necessary to produce complete inhibition in the absence of antibiotics, the stems in both experimental groups all regenerated at the same rate as the control stems. In experiment B, the hydranth density was over four times that necessary to produce complete inhibition without antibiotics. The preparation agitated at this hydranth density with antibiotics (B4) produced some delay in the rate of regeneration. This delay may have been due to the cytolysis of some of the hydranth tissue releasing the same substances present in hydranth extracts (see discussion).

These experiments indicate that in the presence of penicillin and streptomycin in concentrations which suppress bacterial growth, inhibitor water cannot be collected. However, it may be argued (Tweedell, 1958) that the antibiotics used either prevent the hydranths from producing an inhibitor of regeneration or destroy this inhibitor as it is produced. Three observations appear to exclude these alternatives. (1) That penicillin and streptomycin do not destroy the inhibitors was demonstrated by experiments in which bacteria were removed by centrifugation and these antibiotics added to inhibitor water after preparation. In one such experiment, there was no measurable reduction in the activity of the preparation when antibiotics were added (Table II, C3); in another (not listed) there was a slight reduction but not an elimination of the inhibition produced by the preparation (similar to that observed in other preparations when they were sterile filtered). (2) Particularly important are three experiments with penicillin and streptomycin and one with sulfadiazine in which bacterial growth occurred in the preparations even though antibiotic was added at the beginning of aeration. Presumably bacteria resistant to the antibiotics used developed in these preparations. In these cases, the preparations inhibited regeneration in proportion to the amount of bacterial growth which occurred in them (*e.g.*, Table II, C5, 6), showing that, in spite of the antibiotics, if bacterial growth occurred, an active regeneration inhibitor was produced. (3) Three antibiotics—penicillin, streptomycin and sulfadiazine—differing greatly in chemical structure and presumed mode of action, were used alone or in pairs to maintain bacteriostasis. Regardless of which antibiotic was used, if bacterial growth was prevented the preparation failed to inhibit regeneration.

TABLE I

The number of bacteria present in five similar preparations of inhibitor water and the effect of these preparations on regeneration. Observations were made at intervals for 4.3 days

Preparation	Bacteria/ml. × 10^8	Effect of preparation on stems
Control	0.0	10/10 emerged within 2.4 days
1	2.3	10/10 did not begin regeneration
2	3.3	10/10 did not begin regeneration
3	3.7	10/10 disintegrated within 3.6 days
4	5.5	10/10 disintegrated within 1.4 days
5	7.4	10/10 disintegrated within 1.0 day

To see if hydranths were a necessary component of the system, experiments were done in which bacterial growth was allowed to occur in sea water in the absence of hydranths. Dilute proteose-peptone solutions in sea water, aerated for 24 hours, and then sterilized by millipore filtration followed by the addition of antibiotic, were potent inhibitors of regeneration, while control solutions in which bacterial growth was prevented by the addition of antibiotic at the beginning of aeration, at most, slightly retarded regeneration (*e.g.*, Table II, C7, 8; compare C1, 2).

TABLE II

Selected experiments which illustrate the activity of inhibitor water prepared with penicillin and streptomycin. Bacterial density was either estimated (number represented by pluses in the table) or counted directly using a bacteria counting slide (represented by number per ml.). Abbreviations: pen., penicillin; strep., streptomycin

Experiment	Components added to sea water	Bacteria per ml.	Stems regenerated vs. total	Mean time of emergence in days
A1	Pen. and strep.	—	10/10	2.5
2	4 hydranths/ml. + pen. and strep.	–	10/10	2.6
3	4 hydranths/ml. + pen. and strep.	–	10/10	2.5
B1	None	–	10/10	2.3
2	8 hydranths/ml.	+++	0/10	–
3	Pen. and strep.	–	10/10	2.4
4	8 hydranths/ml. + pen. and strep.	–	10/10	3.1
C1	None	ca. 10^5	10/10	2.4
2	2 hydranths/ml.	5×10^8	0/10	–
3	2 hydranths/ml., pen. and strep. added after aeration*		0/10	—
4	Pen. and strep.	$< 10^5$	10/10	2.3
5	2 hydranths/ml. + pen.	2×10^7	5/10	4.8
6	2 hydranths/ml. + strep.	1×10^8	2/10	2.3
7	0.1% proteose peptone + pen. and strep.	ca. 10^5	10/10	3.1
8	0.1% proteose peptone, pen. and strep. added after aeration	3×10^8	0/10	—

* Penicillin and streptomycin were added to a portion of solution C2.

To make certain that the inhibition produced as a result of bacterial growth was not dependent on the presence of specific bacteria, preparations were made using *Escherichia coli.* Cultures were grown in a minimal medium (Davis and Mingioli, 1950) from a small inoculum to 10^9 cells per ml. The bacteria were removed by centrifugation, and the used medium diluted 1:5 in sea water containing antibiotic. Such a preparation completely inhibited regeneration, while control stems placed in a 1:5 dilution of sterile minimal medium with antibiotic regenerated normally.

From these data it is clear that the activity of inhibitor water can be explained on the basis of the bacterial growth which occurs in the medium, and that no other inhibitors can be collected when bacteriostasis is maintained with antibiotics.

TABLE III

Summary of all experiments which indicate that inhibitor water is a by-product of bacterial growth. Refer to the text for explanations of each experiment. Abbreviations: pen., penicillin; strep., streptomycin; sulfa., sulfadiazine

Components added to sea water before aeration	Number of experiments	Bacterial growth	Inhibition of regeneration
None	17	−	−
Hydranths	17	+	+
Hydranths*	6	+	+
Hydranths**	2	+	+
Heat-killed hydranths	4	+	+
Pen., strep., or sulfa.	14	−	−
Hydranths + pen., strep., or both pen. and strep.	7	−	−
	3	+	+
Hydranths + sulfa.	2	−	−
	1	+	+
Stem lengths	2	−	−
Proteose peptone, pen. and strep.	3	−	−
Proteose peptone**	4	+	+

* Preparation sterile filtered after aeration.

** Preparation centrifuged after aeration, penicillin and streptomycin added to the supernatant.

As an argument for the specific role of hydranth structures in producing inhibitor water it has been noted that a population of stems, aerated in sea water, does not produce an inhibitor (Tweedell, 1958). After cutting, the ends of a stem rapidly heal and secrete a thin layer of perisarc, so that very soon a cut stem is entirely covered with chitin. Since no tissue is exposed, a preparation of stems could not be expected to be a good medium for bacterial growth, and this might be the reason why no inhibitor was produced. Experiments were done in which populations of clean stems were cut, washed, and aerated in sea water. Such preparations did not support the growth of significant numbers of bacteria, and, when tested on stems, permitted regeneration at the same rate as the controls.

A summary of the experiments which have been described, together with the number of cases of each type, is presented in Table III. Cases in which very slight bacterial growth occurred in the preparations or in which the preparations only produced a slight delay in regeneration (such as case B4, Table II) are recorded as negative (−) in the table; only definite cases of bacterial growth or regeneration inhibition are recorded as positive (+). As the table indicates, the inhibition of regeneration was always correlated with the growth of bacteria.

It is pertinent to mention certain experiments done with the regeneration inhibitor found in *Tubularia* hydranth extracts. Such extracts were prepared by homogenizing a population of adult hydranths and collecting the supernatant, as described by Tardent (1955) and Tweedell (1958). It was found that the inhibition of regeneration produced by such extracts was not a result of bacterial growth, in that when penicillin and streptomycin were added to the extracts to maintain bacteriostasis the activity of the extracts was not affected in terms of the proportion of stems inhibited by a given dilution of extract. It was found, however, that in contrast to the original report of Tardent (1955), the inhibition produced by *Tubularia* tissue extracts was not specific to hydranth tissue. The supernatant of homogenates from equivalent quantities of stem tissue also suppressed the regeneration of stems. Tardent (personal communication) has obtained the same result recently with *Tubularia larynx*. Preliminary comparisons on a wet weight basis indicate that hydranth tissue is about twice as active a source of inhibitor as stem tissue. The lack of specificity of this inhibitor makes it impossible, however, in the absence of further data, to adequately evaluate the normal physiological role of the substances involved.

Discussion

The results of the experiments with inhibitor water may be summarized as follows. (1) Hydranths agitated in sea water produce bacterial growth and inhibitors of regeneration. (2) If bacterial growth is suppressed with antibiotics, regeneration inhibitors cannot be collected. (3) If antibotics are added at the beginning of aeration but bacterial growth is not prevented, inhibitors can be collected. (4) Bacterial growth in the absence of hydranths produces regeneration inhibitors. These results, together with the appropriate controls, demonstrate that inhibitor water as prepared in these experiments is a by-product of bacterial growth for which the hydranths serve as inoculum and nutrient source. The results, however, should not be taken to indicate that hydranths cannot produce any inhibitors of regeneration, but rather that inhibitor water prepared as described by previous workers contained no inhibitors which could not be accounted for as the products of bacterial rather than hydranth metabolism.

If hydranths are agitated with antibiotics at densities several-fold higher than those used to prepare inhibitor water (*cf.* Tweedell, 1958), occasionally such preparations (*e.g.*, Table II, B4) retard regeneration even though bacteriostasis has been maintained with antibiotics. It is interesting to note that in such cases bulbous outgrowths appear at one or both ends of many of the stems. These outgrowths are similar to those found in stems placed in *Tubularia* tissue extracts (Tweedell, 1958; author's unpublished observations), suggesting that the cytolysis of some of the hydranth tissue has released the substances found in hydranth extract into the water.

Since this manuscript was originally submitted for publication, a paper by Tweedell (1958) has appeared in which the results described in the present paper are discussed. The results of this work were presented incompletely by Tweedell; the results as presented here answer the objections raised in his discussion. In particular, the possibility that the antibiotics used had significant effects other than that of maintaining bacteriostasis has been excluded by the results described above.

Tweedell notes that although bacteria were removed from some of his preparations by sterile filtration the preparations still inhibited regeneration. It is clear from the present work that it is not the bacteria themselves, but rather the metabolites they release into the medium, which are primarily responsible for the activity of inhibitor water. Removal of the bacteria from inhibitor water or proteose-peptone solutions after aeration by filtration or centrifugation, or the addition of penicillin and streptomycin to such preparations, in some cases reduced the inhibitory activity of the preparation but in no case eliminated it.

SUMMARY

1. Rose and Rose (1941) found that adult *Tubularia* hydranths agitated in sea water produced a solution, inhibitor water, which prevented regeneration. They and subsequent workers have ascribed to this inhibitor a role in normal physiological dominance. In the present investigation it has been found that considerable bacterial growth occurs in the solution during the preparation of inhibitor water by the usual methods, and that when antibiotics have been added to maintain bacteriostasis no inhibitor can be collected. Experiments have excluded the possibilities that the antibiotics used are preventing the production of the inhibitor or destroying it as it is produced. It has been shown that metabolites produced by bacterial growth in the absence of hydranths inhibit regeneration.

2. These data lead to the conclusion that inhibitor water represents the byproducts of bacterial growth for which the hydranths serve as source of inoculum and as nutritive medium.

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STUDIES ON THE STRUCTURE AND PHYSIOLOGY OF THE FLIGHT MUSCLES OF BIRDS. 4. OBSERVATIONS ON THE FIBER ARCHITECTURE OF THE PECTORALIS MAJOR MUSCLE OF THE PIGEON

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Denny-Brown (1929), studying the red and white muscles of vertebrates, made some observations on the "light" and "dark" muscle fibers in the breast muscle of the pigeon. The later works on these two types of fibers have been reviewed by George and Naik (1957). More recently, George and Naik (1958a, 1958b) have shown that the red narrow fibers are rich in fat and mitochondria in sharp contrast to the white, broad, glycogen-loaded fibers, which contain only a negligible amount of fat and mitochondria. George and Scaria (1958a) histochemically demonstrated higher lipase activity in the red narrow fibers. The Krebs' cycle enzymes, too, seem to be localized in the narrow fibers (George and Scaria, 1958b). These findings have stimulated considerable interest and called for a basic understanding of the nature and disposition of the fiber components of this muscle as a whole. The present study, therefore, is an attempt to provide a comprehensive picture of the pattern of fiber distribution and the nature of the metabolite load in the different regions of the muscle.

MATERIALS AND METHODS

In order to obtain uniformly well developed *pectoralis major* muscle, only fully grown wild pigeons, either shot or trapped from a single locality, were used throughout for the present study.

Mapping the distribution of the two types of fibers in the muscle

Due to the bipectinate arrangement of the fasciculi, it was found convenient to divide the muscle into twelve regions, each one extending to 10 mm. in length along a hypothetical line, drawn midway between the origin of the muscle fasciculi and the centrally placed tendon (as shown in Fig. 1). From each of these regions at the level of the aforesaid line, fresh frozen transverse sections were cut on a freezing microtome. Subsequently the sections were treated in the following manner. Transferring a fresh frozen section into distilled water or even saline or isotonic sucrose solution resulted in uneven curling up of the section. Again, the size of the muscle piece handled being large, some difficulties which were encountered in the beginning in obtaining a good entire section, were completely avoided by transferring the section directly into chilled 50% glycerol and mounting it on a microslide in the glycerol solution. In the preparations thus made the arrangement of the fibers in the section, however large, was faithfully maintained with no distortions taking place. The glycerol-impregnated sections were thus found to be ideal to manipulate. Moreover, the sections left in glycerol solution and maintained at 0° C. can remain for more than a week without any perceptible defect and thus could be utilized for future observations.

The desired region of the mounted section was projected on the screen of a microphotographic camera at a magnification of 47 × and the photographic printing paper exposed directly to the image. "Normal" bromide papers were found suitable. Using the sliding vernier on the stage of the microscope, continuous photographic records of the distribution of the broad fibers were made (Fig. 5). From such records by the method of random sampling, the mean value of the number of broad fibers per square mm. was determined for every mm. depth of the muscle. A survey of all the twelve regions was thus completed and a graph plotted illustrating the continuous distribution of broad fibers per square mm. at the distance of every 5 mm. along the line 0–120 (Fig. 1). The lines demarcating the areas containing 30–50, 50–70, 70–90, 90–100, 100–120 and 120–140 and 120–150 broad fibers per square mm. were drawn. The entire procedure was

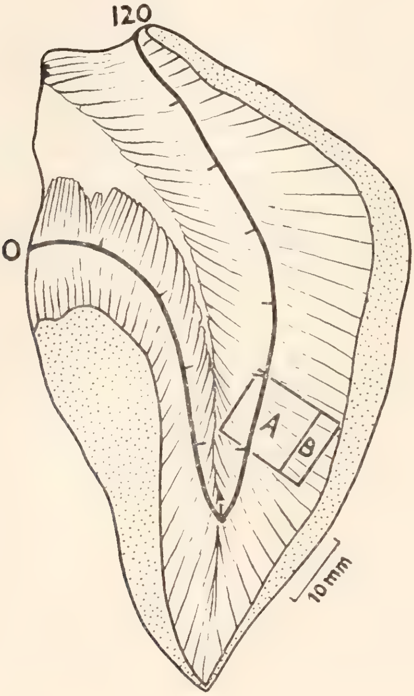

FIGURE 1. Dorsal view of the *pectoralis major* muscle of the pigeon showing the hypothetical lines 0–120 along which the distribution of broad fibers is recorded in Figure 2. The squares A and B indicate the regions of the muscle used for studying the variation in metabolite load and the structure at different depths of the muscle.

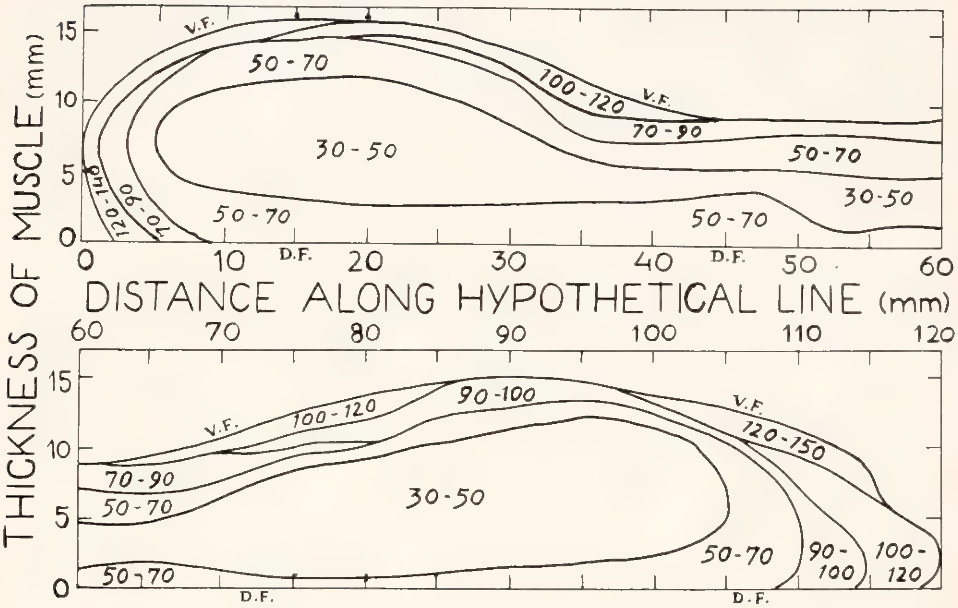

Figure 2. Cross-sectional view of the *pectoralis major* along the line 0–120 drawn in Figure 1. The figures in the chart show the number of broad fibers per square mm. D.F., dorsal face of the muscle; V.F., ventral face of the muscle.

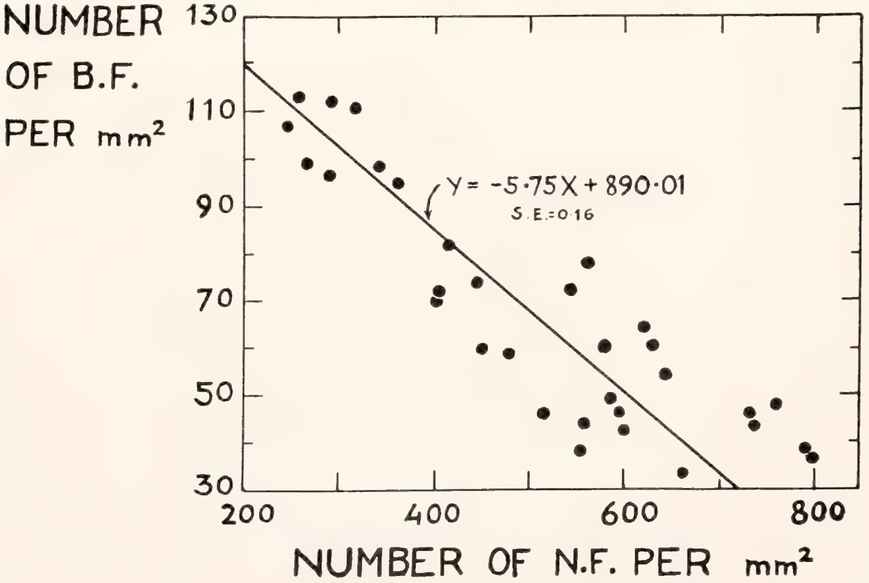

Figure 3. Relation between the number of broad fibers and the number of narrow fibers per square mm. of transverse section of the muscle.

repeated on the *pectoralis* of three pigeons. The results obtained are summarized in a graphical representation as shown in Figure 2. Since the individual variations in the *pectoralis* of different pigeons are considerable, the lines demarcating different areas in the figure are not claimed to be absolute, but they do show the generalized pattern of the distribution of the broad fibers in the *pectoralis major* muscle of the pigeon.

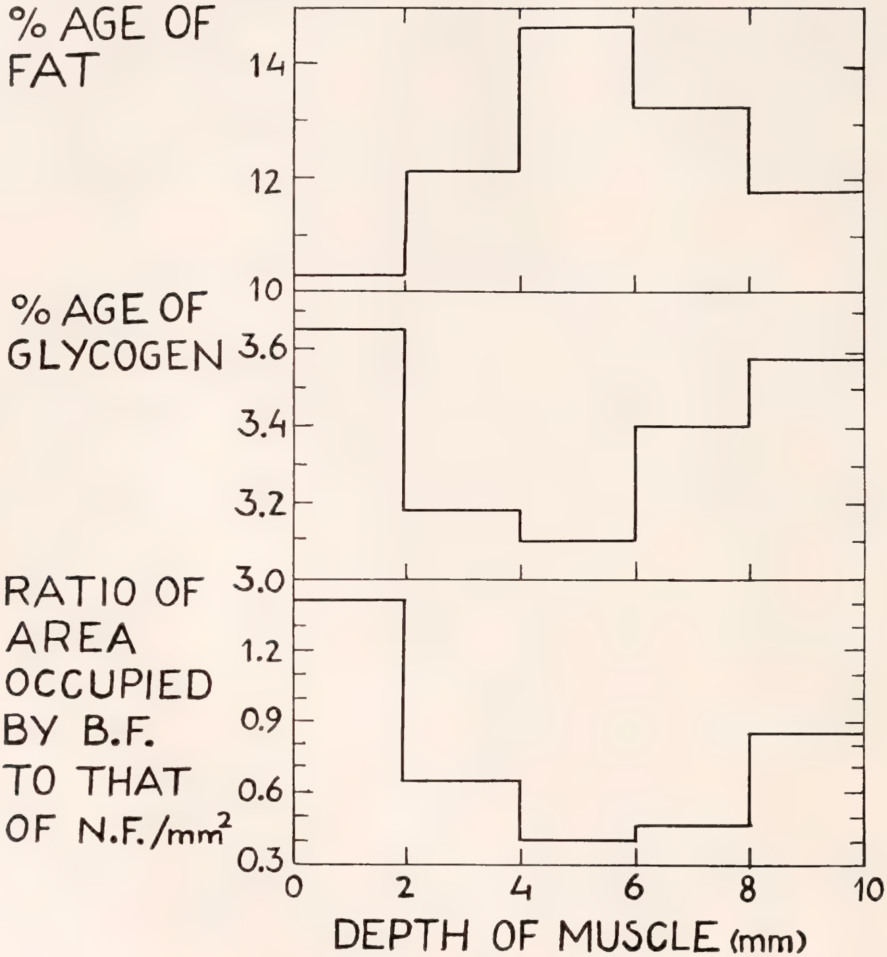

FIGURE 4. Variation in the percentage of glycogen and fat, in relation to the ratio of the area occupied by the broad fibers to that of the narrow fibers per square mm., at different depths of the muscle. The regions of the muscle marked A and B in Figure 1 were used.

For counting the broad as well as the narrow fibers in one and the same region, the same procedure was adopted, except that the image of the section projected on the screen was magnified to about a hundred times, and the sections from the different typical regions of the muscle were used.

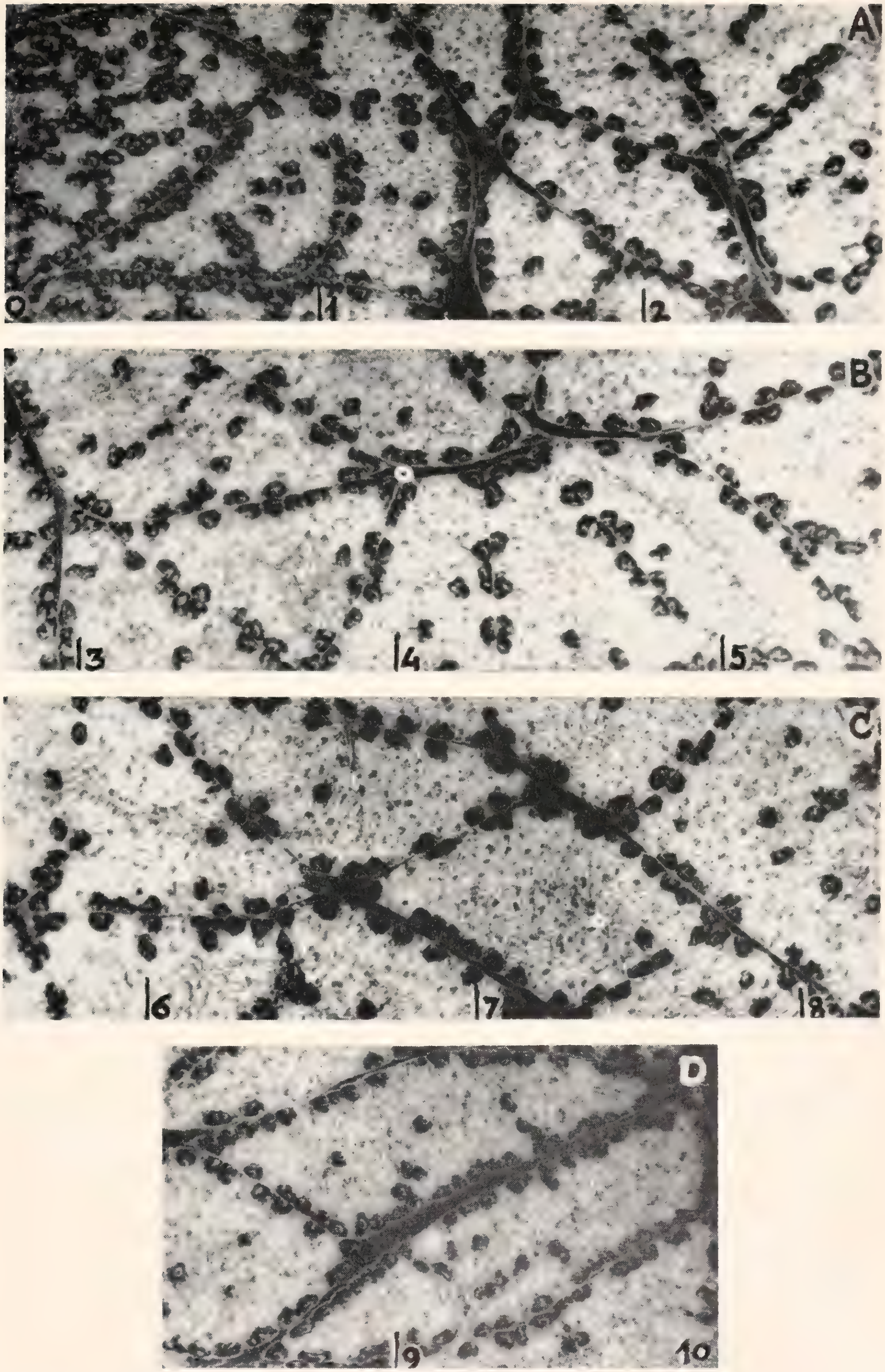

FIGURE 5. Negative prints of the transverse section taken from the region A (Fig. 1) showing the continuous distribution of broad fibers (darker in color) at different depths of the muscle. The numbers 1–10 on microphotographs indicate the depth in mm. from the ventral to the dorsal face of the muscle.

Estimation of fat and glycogen at different depths of the muscle

For the sake of convenience, the region of the muscle (marked A in Fig. 1) on the posteriormost end of the keel was used throughout. In this region the thickness of the muscle is only about 10 mm. and the variation in the distribution of the broad fibers at the different depths of the muscle is gradual. From this region A, a piece about 10 cubic mm. in size was cut out for the estimation of glycogen and a somewhat bigger piece for the estimation of fat. From a region B lateral to A, another piece was cut out and transferred to the freezing chamber of the refrigerator and used later on for studying the distribution of the broad fibers in this region by the method already described.

The muscle piece cut out from region A was mounted on the stage of a freezing microtome so as to obtain horizontal sections. It was frozen hard, the outermost epimysium was peeled off with a pointed forceps or sliced off by a superficial stroke of the microtome knife, and 1-mm. thick slices of the muscle were serially cut. Since all these horizontally cut sections were of uniform and known thickness, each could be said to represent the nature of the muscle tissue at a known depth. The thickness of the sections was not actually measured since the microtome used was a brand new "Sartorius" model and all the possible precautions, such as avoiding the fluctuations in the temperature, were taken so as to obtain sections of uniform and accurate thickness. Each frozen section was immediately transferred to a weighing bottle and dehydrated. The sections to be used for the estimation of glycogen were dehydrated in a vacuum-desiccator at one atmosphere pressure and maintained at 0° C., whereas for fat extraction, sections were dehydrated in an air-oven at 80° C., and finally in vacuum.

The dehydrated sections were weighed and their glycogen content was estimated according to the method of Kemp *et al.* (1954). For the quantity of the muscle used for estimation (about 20–30 mg. per dry weight) it was found necessary to dilute the glycogen extract in the deproteinizing solution to 10 ml. The color developed was measured on the Beckman spectrophotometer (DU model) at 520 μ. For the estimation of fat the dehydrated material was ground and, after weighing, transferred to a fat-extraction thimble. The fat was extracted in the Soxhlet apparatus with 1:1 ethanol-ether mixture (George and Jyoti, 1955). About 70–100 mg. of dehydrated muscle were used for each estimation.

The estimation of glycogen in the two types of fibers

Small pieces from the breast muscle of a decapitated pigeon were cut out and dropped in previously chilled 80% methanol and left undisturbed at − 10° C. for 24 hours. The fibers from the muscle thus preserved were teased out in methanol under a binocular dissection microscope with watch-maker's forceps. The two types of fibers were isolated and transferred to two separate containers containing methanol and fitted with air-tight glass lids and stored in the refrigerator. Sufficient numbers of fibers which would yield about 2–5 mg. in dry weight were isolated and collected for each estimation. These fibers were then removed from the methanol solution, dehydrated in vacuum and weighed on a microbalance. Glycogen was estimated, as already mentioned, by the micromethod of Kemp *et al.* (1954).

Results

Figure 5 presents a typical picture of the distribution of broad fibers in the muscle. In each fasciculus the broad fibers are mainly concentrated towards the periphery. This pattern is maintained throughout the muscle. In regions of the muscle where there are larger numbers of broad fibers or lesser numbers of narrow fibers, the fasciculi have a smaller cross-sectional area with broad fibers closely packed along their borders without any intervening narrow fibers. The number of broad fibers per square mm. in the different regions of the muscle is shown in Figure 2. The relation of the number of broad fibers to that of the narrow ones per square mm. is shown in Figure 3. From both these, the number of broad fibers, as well as the number of narrow fibers per square mm., in any region of the muscle could be approximately determined.

The variation in the metabolite load and the number of broad fibers per square mm. at different depths of the muscle are indicated in Table I, while in Figure 3 the same data are utilized to show the relation between the structure of the muscle and the metabolite load. The number of narrow fibers for the corresponding number of broad fibers was calculated by using the formula of the regression line in Figure 3 and the ratio of the area occupied by the broad fibers to that of the narrow fibers in square mm. was determined by using the mean value of the diameter of these fibers. The diameter of the broad fibers is 69.00 ± 14.00 μ (1000) and that of the narrow fibers is 30.11 ± 6.56 μ (2000). The figures given in parentheses indicate the number of fibers measured from the fresh frozen sections taken from the various regions of the muscle.

Table I

The number of broad fibers per square mm. and the percentage of fat and glycogen at different depths of the breast muscle of the pigeon. (The portion of the muscle marked A in Fig. 1 was used. The figures indicate the average values of six sets of readings)

Depth of the muscle in mm. (starting from the ventral face)	Number of broad fibers per square mm. ±S.D.	Percentage per dry weight of the muscle ±S.D.	
		Glycogen	Fat
0–2	90 ± 14	3.655 ± 0.275	10.289 ± 1.942
2–4	63 ± 8	3.175 ± 0.054	12.095 ± 1.056
4–6	48 ± 3	3.102 ± 0.127	14.632 ± 1.752
6–8	51 ± 4	3.409 ± 0.184	13.250 ± 0.571
8–10	72 ± 9	3.588 ± 0.236	11.743 ± 0.572

The values of the glycogen content of the broad and narrow fibers, calculated on the dry weight of the muscle preserved in methanol, are, respectively, 10.240 ± 0.093% and 2.464 ± 0.311% (each value is the mean of three readings). Methanol removes much of the fat (mainly from the narrow fibers) and some of the amino acids.

Discussion

It has been known that in many active muscles, the muscle fibers towards the periphery become larger in diameter and lighter in color, compared to those in the interior. In such muscles even in the individual fasciculus, the light fibers are situated towards the periphery. In the pigeon breast muscle, the white broad fibers and the red narrow fibers show a somewhat similar distribution pattern but these fibers differ from the light and dark fibers of the other muscles in that they are sharply differentiated into two distinct types without any intermediate forms. The broad fibers are glycogen-loaded and poor in fat inclusions and mitochondria, whereas the narrow fibers are fat-loaded and have a high mitochondrial content and are poor in glycogen (George and Naik, 1958a, 1958b).

In a single muscle uneven distribution of metabolites has been long since realized. To reduce such localized variation to the minimum, customarily a large piece of muscle is utilized for the estimation of metabolites. Present work shows that in a muscle like the *pectoralis major* of pigeon having heterogeneous cellular elements, variation in metabolites in the different regions of the same muscle and even in a single fasciculus is quite large. Needless to say, what applies to glycogen and fat might equally apply to other chemical constituents in which the two types of fibers differ.

A general belief that the muscle fibers towards the periphery of the muscle are more active than those in the interior and, due to higher activity, increase in diameter, does not seem to hold good, at least in the case of the *pectoralis* of pigeon. Undoubtedly, the red fibers of pigeon breast muscle, due to their remarkably well developed enzyme systems, play a major role in effecting the sustained contractions of the muscle. In white fibers, on the other hand, the oxidative processes are not developed or developed only to a negligible extent, in that the dehydrogenase activity in these fibers, as shown by histochemical method, is negligible or nil (George and Scaria, 1958b). All the same, the white fibers are not inactive elements of the pigeon breast muscle. In the normal animal they show no signs of atrophy. A glycerinated white fiber of pigeon breast muscle contracts in the same manner as a glycerinated red fiber of the same muscle on the addition of ATP. The study on the reactions of these two types of fibers to experimentally induced disuse atrophy has yielded significant results. When the movement of the humerus is restricted for three months by a plaster cast, the white fibers in the deeper layer of the muscle show acute sign of atrophy whereas the red fibers appear practically unaffected (George and Naik, unpublished data). These findings suggest the possibility of some differences in the mechanical properties of the two types of the fibers and in that case some physical factors may underlie the distribution pattern of the two types of fibers in the muscle.

Denny-Brown (1954), has shown that a single nerve in the breast muscle of pigeon can innervate both, the red as well as the white fibers. Since the activity of these muscle fibers must be conditioned by the fundamentally different chemical system in them, it is difficult to believe that the amount and the mode of activity performed by these two types of fibers are the same. In what exact manner the white fibers contribute to the activity of the muscle is far from clear and as a prelude to such an understanding, an extensive study of these fibers is essential. For such a study Figure 2 can be a useful guide. Moreover, the method used in the present work to study the variation in the metabolite load in relation to the variation in the fiber make-up of the muscle, can be used for studying the distribution of various constituents such as enzymes, amino acids and minerals in the muscle.

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SUMMARY

1. The relative distribution pattern of the red and white muscle fibers in the breast muscle of the pigeon is studied.

2. There exists a direct relation between the distribution of metabolites and that of the two types of fibers in the different regions of the muscle.

3. Quantitative estimation of glycogen in the two types of fibers confirms the higher concentration of glycogen in the white fibers.

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THE EFFECT OF OSMOTIC STRESS ON THE IONIC EXCHANGE OF A SHORE CRAB

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The decapod Crustacea have received considerable attention with regard to their ability to regulate the inorganic ions of their blood (Krogh, 1939; Robertson, 1949, 1953, 1957; Prosser *et al.*, 1950). Prosser *et al.* (1955) studied responses of the shore crab *Pachygrapsus crassipes* to different concentrations of sea water. The chief concern of their study was to determine the changes in the ionic concentrations of blood and urine which were effected by altering the concentration of the external medium from normal. Determinations on the total losses and gains of the respective ions between animal and medium were not made nor were the effects of desiccation on ion concentrations in urine or blood determined. This information would be of special interest in the case of a semi-terrestrial crab such as *Pachygrapsus*.

Gross (1958) demonstrated that when *Pachygrapsus crassipes* was placed under osmotic stress, the principal exchanges of potassium were between the medium and a source of potassium other than the blood, not mainly between blood and external medium. Also, evidence was produced that an extra-vascular pool participates in sodium exchanges between crab and medium. This paper will produce further evidence that extra-vascular salt pools in *Pachygrapsus* contribute to ionic exchanges with the medium, special attention being paid to calcium and magnesium. The effects of desiccation on the ionic concentration of urine and blood in *Pachygrapsus* will be revealed and data confirming the findings of Prosser *et al.* (1955) will be produced.

Material and Methods

The subject of this investigation, *Pachygrapsus crassipes* Randall, was collected at Laguna and Dana Point, California. All specimens were between molts, and were mature, none being smaller than 20 gm.

Urine was sampled by inserting a micropipette into the excretory pores. Blood was obtained by puncturing the cuticle of the leg joints with a micropipette. Sodium and potassium were measured by means of a Beckman flame photometer. Urine and blood were measured and diluted appropriately before being used directly in the flame photometer (Gross, 1958). Samples as small as 0.05 ml. thus could be analyzed to an accuracy of 2% for sodium and 10% for potassium at the minimum concentrations measured in this investigation. Thus, before and after treatment samples of blood from the same crab could be analyzed for sodium and potassium. Calcium and magnesium were determined by titration with ethylenediamine tetra acetic acid (EDTA) a method described by Schwarzenbach *et al.* (1946) and Knight (1951). This method requires about 0.25 ml. of urine and about 0.50 ml. of blood. Because of the relatively large volume needed, repeated blood samples on the same specimen were not taken. Urine samples were diluted to 100 ml. and titrated directly. Media were titrated directly. Blood samples were dialyzed against distilled water and the dialysate was titrated, a correction being applied for the content of the dialysis bag. This process, which was not necessary in the case of urine, gave a more distinct end-point than titrating the diluted blood directly. Calcium and magnesium thus could be recovered within an accuracy of 5% for the minimum concentrations measured.

TABLE I

Effects of stress on ionic concentrations of blood and urine in Pachygrapsus

	Treatment											
	50% sea water			100% sea water			150% sea water			Desiccation		
	Mean	S.D.	No. crabs	Mean	S.D.	No. crabs	Mean	S.D.	No. crabs	Mean	S.D.	No. crabs
Sodium (mEq./l.)												
Blood	397	24	37	*483	17.3	36	582	34	30	536	27.4	32
Urine	380	60	37	378	64.0	15	353	106	30	297	104	15
U/B ratio	0.96	0.14	37	0.78	0.14	15	0.63	0.16	30	0.56	0.17	15
Medium	232			464			696					
Potassium (mEq./l.)												
Blood	7.36	1.4	37	*7.43	0.72	36	10.23	1.48	30	11.5	1.63	32
Urine	9.95	3.5	37	7.76	1.35	15	9.59	1.13	30	14.8	3.18	15
U/B ratio	1.45	0.50	37	0.82	0.19	15	0.94	0.33	30	1.34	0.50	15
Medium	4.9			9.8			14.7					
Calcium (mEq./l.)												
Blood	34.8	7.9	24	29.6	5.9	44	36.4	4.8	30	45.2	10.7	36
Urine	32.7	7.1	31	36.0	6.3	15	47.9	5.2	20	44.4	7.36	12
U/B ratio	0.98	0.13	23	1.17	0.20	15	1.33	0.18	29	1.07	0.33	12
Medium	10.0			20.0			30.0					
Magnesium (mEq./l.)												
Blood	13.6	5.36	24	20.0	6.1	44	27.1	4.22	30	28.5	15.9	36
Urine	70.5	41.1	31	236	87	15	408	122	29	424	144	12
U/B ratio	5.62	4.52	23	13.6	5.3	15	15.4	4.44	29	23.6	10.9	12
Medium	52.0			104			156					

* Gross (1958).

In order to measure the exchange of ions between animal and external medium, crabs freshly removed from normal sea water were weighed, and blood was sampled for sodium and potassium determinations. The crabs then were rinsed in water of the salinity to which they were to be exposed, then immersed in a small volume (50 ml.) of that same water for a period of about 24 hours. Adequate precautions were taken against water loss by evaporation. Values concerning sodium and potassium exchanges (Table II) have been reported previously (Gross, 1958) and include some data on animals immersed 24–48 hours in 100 ml. The crabs could raise themselves out of the water and therefore were not completely immersed at all times. After a period of about 24 hours, the animals were removed from the media and their blood and urine sampled for the analysis of sodium, potassium, calcium and magnesium. Likewise the media were analyzed for these ions.

Other crabs freshly removed from normal sea water were weighed, then desiccated for a period of about 48 hours for a loss of about 7% original weight. After this treatment the blood and urine were analyzed for the above four cations. Analyses of blood potassium and sodium were made before and after desiccation on individual crabs.

Table II

Relative ion changes in blood and external medium caused by altering external medium from normal

	Mean of ratios*	50% sea water	S.D.	No. crabs	150% sea water	S.D.	No. crabs
Na	$\frac{\text{Blood change (mEq./l.)}}{\text{Medium change (mEq./l.)}}$	2.56	0.82	28	2.63	0.80	25
K	$\frac{\text{Blood change (mEq./l.)}}{\text{Medium change (mEq./l.)}}$	0.56	0.43	20	1.00	0.70	24
Ca	$\frac{\text{Blood change (mEq./l.)**}}{\text{Medium change (mEq./l.)}}$	0.93	3.23	22	0.78	0.66	28
Mg	$\frac{\text{Blood change (mEq./l.)**}}{\text{Medium change (mEq./l.)}}$	0.85	0.89	24	0.64	0.40	26

* Change in medium for all ions is corrected to a volume equal to the weight of the crab.

** Blood change for calcium and magnesium equals the difference between mean of normal crabs and the observed blood concentration after treatment for each crab. Medium change is the observed concentration change in the medium after treatment for each crab.

RESULTS

Table I presents the urine and blood concentrations of sodium, potassium, calcium, and magnesium after the following treatments: a) immersion in normal sea water; b) immersion in 50% sea water; c) immersion in 150% sea water and d) desiccation for a water loss of about 7% body weight. Comparing the blood values after immersion in 100% sea water with those of Prosser *et al.* (1955), sodium and calcium appear in agreement. However, the potassium (7.43 mEq./l.) and magnesium (20.0 mEq./l.) values are considerably less than those reported by the above workers (12.1 mEq./l. and 58.4 mEq./l., respectively). On the other hand Schlatter (1941) reported blood ion concentrations for this same species which agree closely with the values of the present investigation.

It should be emphasized that the indicated stress media (Table I) represent only the initial sea water concentrations, and that these necessarily were altered by exchanges of salts with the animal. However, an accurate knowledge of the sustained osmotic gradient and the final blood concentrations is of little meaning in this investigation, since as described above, the animals were able to raise themselves out of the water. The main objectives of this study are to demonstrate: 1) the degree to which a blood ion change is reflected in the external medium and 2) the role of the antennary glands in controlling the ion content of the animal. It also should be pointed out that in this crab alterations in the blood concentration in aqueous media are effected by salt exchanges, not water (Gross, 1957).

Data in Table I, however, do reveal something of the ability of *Pachygrapsus* to regulate ions in the different sea water concentrations. Thus blood sodium is held above the sodium concentration of the dilute medium and normal sea water, but below the concentration of the hypertonic medium. Blood potassium is held above the concentration of the dilute medium, but below the concentration of normal sea water or the concentrated medium. Gross (1958) reported that when *Pachygrapsus* was immersed in a small volume of 50% sea water, the blood potassium remained less concentrated than the medium potassium. However, these animals were immersed for longer periods than those reported in the present studies (Table I) during which time the animal lost more potassium and the medium gained potassium. Table I also shows that the blood calcium remains more concentrated than the medium calcium for all treatments. Blood magnesium, on the other hand, is less concentrated than the medium magnesium for all aqueous conditions. All four ions increase under conditions of desiccation.

The ratios, urine concentration/blood concentration (U/B ratio), for each respective ion suggest the role of the antennary glands in the ion regulatory mechanism. Values in Table I are means of U/B ratios observed in individual specimens, not ratios of means. Thus all the mean U/B ratios for sodium are less than one, indicating that the antennary glands do not regulate sodium under this set of conditions. That is, sodium is not eliminated effectively when the gradient between blood and medium favors a gain; nor is it conserved effectively when the gradient favors a loss to the medium (mean U/B ratio in 50% sea water = 0.96).

With respect to potassium the mean U/B ratio is less than one when the crab is immersed in 100% or 150% sea water. Thus the antennary gland does not regulate potassium for this set of conditions. In 50% sea water the mean U/B ratio is 1.45 which means, if anything, potassium is being wasted when it is needed. However, for conditions of desiccation the mean U/B ratio is 1.34 which is significantly greater than one, $P < 0.01$. If then there were sufficient production of urine under conditions of desiccation, the antennary glands would tend to keep the blood concentration of potassium at a normal level.

With respect to calcium the mean U/B ratios for crabs immersed in 50% sea water or subjected to desiccation are not significantly different from unity. Thus the antennary glands are ineffective as regulators of calcium for these two conditions. On the other hand, after immersion in 150% sea water the mean U/B ratio is 1.32 which is significantly different from one, $P < 0.01$. In normal sea water the U/B ratio is 1.17, again being significantly greater than one, $P < 0.01$. Thus, the antennary glands might have a small role in regulating calcium, but in no sense as large a role as they have for magnesium.

Data in Table I demonstrate that the mean U/B ratios for magnesium under all conditions studied are much greater than unity. Even after immersion in 50% sea water, the mean ratio is 5.62. However, it should be pointed out that even in this diluted sea water the gradient between blood and external medium favors the uptake of magnesium. Also, it will be noted that the mean ratio under conditions of desiccation is 23.6 which suggests that the urine concentration depends on the blood concentration, not entirely on the rate of influx from the external medium.

The data presented in Table I concerning the treatments in aqueous media are qualitatively in general agreement with the findings of Prosser *et al.* (1955), particularly with regard to the role of antennary glands in the regulation of magnesium. Quantitatively the data presented in Table I differ somewhat from those reported by Prosser *et al.* (1955). However, precise comparison should not be attempted because of differences in experimental procedure. For example, crabs of the present investigation were immersed directly in small volumes of stress media for a maximum of about 24 hours. The data presented by the above workers were obtained on animals gradually acclimated to osmotic stresses for a period of at least 5 days in relatively large volumes of media.

On the other hand there are certain differences which warrant attention. Normal blood potassium and magnesium differences already have been mentioned above. It will be observed that blood calcium after immersion of the animal in 50% sea water (34.8 mEq./l.) is higher than it is for animals from normal sea water (29.6 mEq./l.). These means are significantly different; $P = 0.01$. Prosser *et al.* (1955) showed decreases in blood calcium in 50% sea water which, of course, would be expected. It was thought that perhaps the increased blood calcium resulting from immersion in dilute sea water was an effect of the small volume of medium. Therefore, blood calcium of crabs immersed in large volumes (about 700 ml.) of 50% sea water for 24 hours was determined. The mean blood calcium of 24 crabs thus treated was 30.9 mEq./l., S.D. = 9.0. This is not significantly different from the mean (34.8) obtained by the other treatment; nor is it significantly different from the average blood calcium of normal crabs. These workers also called attention to the inverse relationship between the urine sodium concentration and the blood sodium concentration. That is, the urine sodium of animals immersed in concentrated sea water was less concentrated than that of animals immersed in normal sea water, which in turn was less concentrated than that of animals immersed in dilute sea water. The means for urine sodium after treatment in the three aqueous media (Table I) cannot be shown to be significantly different, but the U/B ratios do suggest the same phenomenon. That is, the ratios decrease as the animal is placed in increasing concentrations of sea water. These ratios are all significantly different from each other; $P < 0.01$. The U/B ratio for the desiccated crabs is not significantly different from the U/B ratio in crabs exposed to concentrated media, but is significantly different from the ratios obtained for crabs given the other treatments; $P < 0.01$.

Data in Table II demonstrate the ionic changes that occur in the medium when a given change in the blood is effected. The measurement of calcium exchanges with stress media was complicated by the fact that this ion is lost in significant amounts when the animal is immersed in normal sea water. Such was not the case for the other ions. It became necessary, therefore, to apply a correction to the calcium exchanges, based on an average loss to normal sea water by 30 crabs. This amounted to 0.5 mEq./l. per gram of crab for a 24-hour period in 50 ml. of medium. It was thus necessary to assume that this *normal* loss is constant in all concentrations of sea water, an assumption which subjects the values for calcium change in the medium to considerable error.

The values for sodium and potassium have been reported previously (Gross, 1958) and represent means of the ratios, blood change (mEq./l.)/medium change (mEq./l.), in individual crabs where the blood change is the difference between the concentration before treatment and the concentration after treatment. For calcium and magnesium the values in Table II also represent means of the ratios, blood change (mEq./l.)/medium change (mEq. l.), in individual crabs, but since only one sample of blood could be extracted from single specimens for calcium and magnesium determinations, the blood change (mEq. l.) in the ratio for calcium and magnesium equals the difference between the observed blood concentration after treatment and the average blood concentration for crabs from normal sea water.

With respect to sodium, the mean ratios are greater than 2.5 in both 50% and 150% sea water. The response to hypertonic stress and hypotonic stress seems to be symmetrical. With respect to potassium the ratio is unity or less; while it is 0.56 for crabs immersed in 50% sea water, it is 1.00 for crabs immersed in 150% sea water. However when ion exchanges were measured in crabs transferred from 50% to 150% sea water or vice versa, a symmetrical response for potassium exchanges is observed, the mean ratio, change in blood (mEq./l.)/change in medium (mEq./l.), being about unity in both extreme stresses (Gross, 1958).

TABLE III

Ion increase in blood caused by desiccation

	No. crabs	Mean change in concentration (% original) per 1% body weight loss by evaporation	S.D.
Na	84	+2.20	0.71
K	50	+8.68	11.75
Ca	34	+5.47	4.23
Mg	35	+3.87	9.42

The mean ratio for calcium and magnesium is less than one for all treatments. Attention should be called to the large variance for the calcium ratio, following immersion in 50% sea water. It also should be mentioned that the ratio, mean blood change (mEq./l.)/mean medium change (mEq. l.), is $\frac{+5.2}{-1.82} = 2.87$, the signs of the numerator and denominator being opposite to expectation. Not only does the average value for the blood calcium increase after treatment in dilute sea water, but the medium apparently loses rather than gains calcium. The difference between the mean of the ratios (0.93) and the ratio of the means (2.87) can be explained on the basis of the large variance.

Table III reveals ionic changes that occur in the blood when *Pachygrapsus* is desiccated for a loss of about 7% body weight. The sodium and potassium values, again, have been reported previously (Gross, 1958) and represent averages of changes in individual crabs, where the blood concentration change was determined by before- and after-treatment readings on the same individual. The values for calcium and magnesium are means of blood concentration changes for individual crabs, but since only after-treatment blood samples were taken, the blood change for these two ions is represented by the difference between the observed concentration in an animal following desiccation and the mean blood concentration of the respective ions in crabs from normal sea water. In Table III it can be seen that the average change for sodium is less than the values for the other ions. While the potassium and calcium changes are significantly greater than the sodium change, $P < 0.001$, the mean magnesium change cannot be considered significantly different from the sodium change. It will be explained below that blood ions which increase more in concentration than blood sodium probably shift from a salt pool (perhaps the intra-cellular space) into the blood when the animal is desiccated.

DISCUSSION

The ratios, blood change (mEq./l.)/medium change (mEq./l.), presented in Table II suggest that the principal exchanges of potassium, calcium, and magnesium between animal and medium are not ultimately between blood and external medium. A ratio of unity means that the concentration change in an external medium which is equal in volume to the animal is identical to the concentration change in the blood. Of course, much of the animal's volume is isolated from the osmotic and ionic processes which occur in the blood. Thus for a ratio of unity, the actual loss or gain of ions with the medium would be greater than the loss or gain of ions in the blood. Therefore a source other than the blood must be contributing to these exchanges. These ratios also can be expressed as "apparent volume of distribution," using the equation $V = M/P \times 100$ (Gross, 1958) where:

V = "apparent volume of distribution" in % body weight;

$$M = \frac{\text{weight of medium}}{\text{weight of animal}};$$

$$P = \frac{\text{change in blood ion concentration (mEq./l.)}}{\text{change in medium ion concentration (mEq./l.)}}.$$

Thus, the "apparent volume of distribution" for sodium is 38.5% body weight and for potassium, calcium and magnesium more than 100% body weight, which only can be interpreted as an aggregation of these three ions in some sort of pool where they are much more concentrated than they are in the blood. This also means that the extra-vascular pools ultimately contribute more to potassium, calcium and magnesium exchanges with the medium than does the original blood supply (more than twice as much). At least, in the case of potassium, the pool probably lies mainly in the intra-cellular space, because it is well known that intra-cellular potassium concentrations are high. In the crab *Carcinus* the relative muscle concentrations of sodium, potassium, calcium and magnesium are 50, 120, 11 and 32 (mEq./kg. water), respectively (Shaw, 1955). If this were representative of intra-cellular concentrations, it would seem unlikely that the intra-cellular space harbors the pool for magnesium and calcium. Although the nature of the pools is unknown, it becomes apparent that a change of a blood ion concentration can occur without a loss or gain in the medium. Or exchanges between animal and medium can occur without being reflected in the blood. The probable exception to this is sodium. The "apparent volume of distribution" for sodium was calculated to be 38.5% body weight for the moderate stresses of 50% and 150% sea water. Webb (1940) estimates the blood volume of the crab *Carcinus* as 36% body weight. Thus the calculated volume, 38.5% body weight, which seems close to a reasonable value for blood space, means that the major sodium exchanges are between the blood and external medium. Though there is evidence that a sodium pool contributes to such exchanges when the animal is exposed to extreme osmotic stress, its role is relatively small percentage-wise, compared with the other ions (Gross, 1958). On the other hand sodium contributes about half the ions of the blood; thus the small percentage effect of a sodium pool would nevertheless affect significantly the total osmotic pressure of the blood.

Burger (1957) immersed lobsters in media of abnormally high magnesium concentrations and noted that neither the blood nor the urine magnesium elevated. On this evidence he concluded that the animal was impermeable to magnesium. However, he did not consider the possibility that the magnesium could enter the animal and be fixed outside of the vascular system, a phenomenon which obviously occurs in *Pachygrapsus*.

The variance for the mean of the calcium ratios, blood change/medium change, when the stress was 50% sea water is high. Nevertheless this ratio for calcium (0.93) is significantly less than the mean ratio for sodium (2.56), $P < .025$. It should be emphasized that the mean blood calcium after immersion in 50% sea water was more concentrated than that for crabs from normal sea water. Also, the corrected average change for calcium in the medium indicated a loss rather than the expected gain. Now, it was revealed above that crabs in normal sea water tend to lose calcium, and the average loss in normal sea water was applied as a correction to the medium measurements, assuming that a loss of calcium (probably by way of the gut) would be the same in a stress as in a normal medium, but if there were a curtailment of *normal* calcium output in dilute sea water, then the correction would be too large and falsely could make the sign of the change in the medium negative. It should be mentioned that the observed changes in the medium without correction were all positive. If the sign of the corrected medium change is in error, then the increase in the blood calcium concentration after immersion in 50% sea water could be caused only by contributions from a calcium reservoir.

Data in Table III demonstrate that for a given weight loss by evaporation the average increase in the blood sodium concentration is less percentage-wise than the increase for the other ions. It was concluded by Gross (1958) that such a difference in increase between sodium and potassium under conditions of desiccation could not be explained on the basis of sodium exclusion from the blood. Rather, it was concluded that it represented a shift of potassium ions from extra-vascular spaces into the blood space. Data for calcium presented in Table III suggest that the same phenomenon happens in the case of this ion; values for magnesium are questionable. No adaptive significance can be assigned to such a phenomenon; rather it is interpreted as a physiological failure which imposes a limitation on the terrestrial habits of this crab.

The U/B ratios presented in Table I suggest the role of the antennary gland as an ion regulator. It has been established previously (Prosser *et al.*, 1955) that this organ is ineffective as an osmotic regulator. Thus, it seems probable that a principal function of the antennary gland is the regulation of magnesium. That is, the U/B ratio with respect to magnesium is much greater than unity. Yet the effectiveness of the antennary glands as magnesium regulators for each experimental condition cannot be known for certain until the volume of urine production is known for each osmotic situation. Thus, even though the urine magnesium is high when the animal is desiccated, it is possible that little or no urine is produced when the animal is removed from an aqueous medium. Nevertheless, the antennary glands may effectively remove magnesium ions from the blood, thus tending to keep the blood levels normal, even though no ions are ejected from the animal.

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SUMMARY

1. The effects of osmotic stress on the ion concentration in the blood of the crab, *Pachygrapsus crassipes*, were investigated. Stresses imposed were 50% sea water, 150% sea water and desiccation to a water loss of about 7% body weight.

2. The observed ratios, blood change (mEq./l.)/medium change (mEq./l.), for sodium, potassium, calcium and magnesium after the crab was transferred from normal sea water to 50% or 150% sea water yielded values for "apparent volume of distribution." The average value for sodium was 38.5% body weight, but for the other three ions was at least 100% body weight.

3. The large values for "apparent volume of distribution" in the cases of potassium, calcium and magnesium indicate that these ions are contained in extra-vascular pools in greater concentrations than they are in the blood and that these pools participate in ion exchanges between animal and medium. Thus, a concentration change can occur in the blood without being reflected in the medium or vice versa.

4. Calcium is lost to the medium by *Pachygrapsus* when it is immersed in normal sea water. Blood calcium increases when a crab is transferred from normal sea water to dilute sea water.

5. When *Pachygrapsus* is desiccated, the blood concentrations of potassium, calcium and magnesium average greater increases than does the sodium concentration. This suggests that potassium, calcium and possibly magnesium shift from an extra-vascular pool into the blood space. The phenomenon is interpreted as a physiological failure and a factor which may limit the terrestrial life of this species.

6. The ratio, urine concentration (mEq./l.)/blood concentration (mEq./l.), for the respective ions suggests the role of the antennary glands as ion regulators

under the various stress conditions. Thus the antennary glands were found to be relatively ineffective as regulators of sodium, potassium and calcium for all conditions studied. The U/B ratio for magnesium averaged 5.62 when the crab was immersed in 50% sea water; 13.6 for normal sea water; 15.4 for 150% sea water and 23.6 when the crab was desiccated. These high ratios suggest that a principal role of the antennary glands is magnesium regulation.

7. The volumes of urine production which have not been measured must be known before the effectiveness of the antennary glands as magnesium regulators can be determined.

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HISTOLOGY AND METABOLISM OF FROZEN INTERTIDAL ANIMALS[1]

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Many invertebrate animals are normally exposed to environmental temperatures far below the freezing point of their body fluids. Although supercooling may sometimes be a factor in survival (Salt, 1950; Ditman *et al.,* 1942; Scholander *et al.,* 1953), freezing occurs in nature among insects (Asahina *et al.,* 1954; Scholander *et al.,* 1953), shore animals (Kanwisher, 1955), and other groups (Luyet and Gehenio, 1940.) On the shore during winter, for example, freezing and thawing occurs twice a day when the animals are exposed to the cold air by the tide. Intertidal animals in the Arctic may be frozen for as long as 6 months (Kanwisher, 1955). The survival of these animals depends on their being able to have most of their body water turned to ice. It is remarkable that no injury is produced in a living system when more than half of its bulk is changed to a crystalline solid. I am reporting here some investigations on the histology and metabolism of these intertidal animals.

HISTOLOGY

In the freeze-drying histological technique, tissue is cooled very quickly with liquid nitrogen. Freezing occurs so fast that ice crystals do not have time to grow very large and cellular organization is very little disturbed. The water is removed by vacuum while the sample is kept cold. The resulting dehydrated tissue matrix is imbedded, sectioned, and stained in a conventional manner. I have used the method here to capture the situation in tissue from shore animals frozen to relatively mild natural temperatures. Comparison with material from unfrozen animals has shown the distortions caused by the freezing.

Animals were collected from the shore at Woods Hole in January and moved to a −10° cold room without thawing. Sections of tissue about 1 mm. thick were cut with a cold knife, held with cold tweezers, and plunged into a vial of isopentane suspended in a container of liquid nitrogen. The isopentane allows a faster heat transfer because it does not boil and form an insulating gas layer. The hard frozen samples were quickly transferred to the already cold dehydrating chamber and vacuum applied for 24 hours at about −45°. The dehydrated tissue was then imbedded in de-gassed paraffin already in the chamber with the vacuum still applied. Photomicrographs of 10-micron sections are shown in Figure 1. The unfrozen controls were tissue taken from identical animals that had thawed at room temperature for an hour.

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[1] Contribution Number 1013 from the Woods Hole Oceanographic Institution. This study was aided by a contract between the Office of Naval Research and the Arctic Institute of North America.

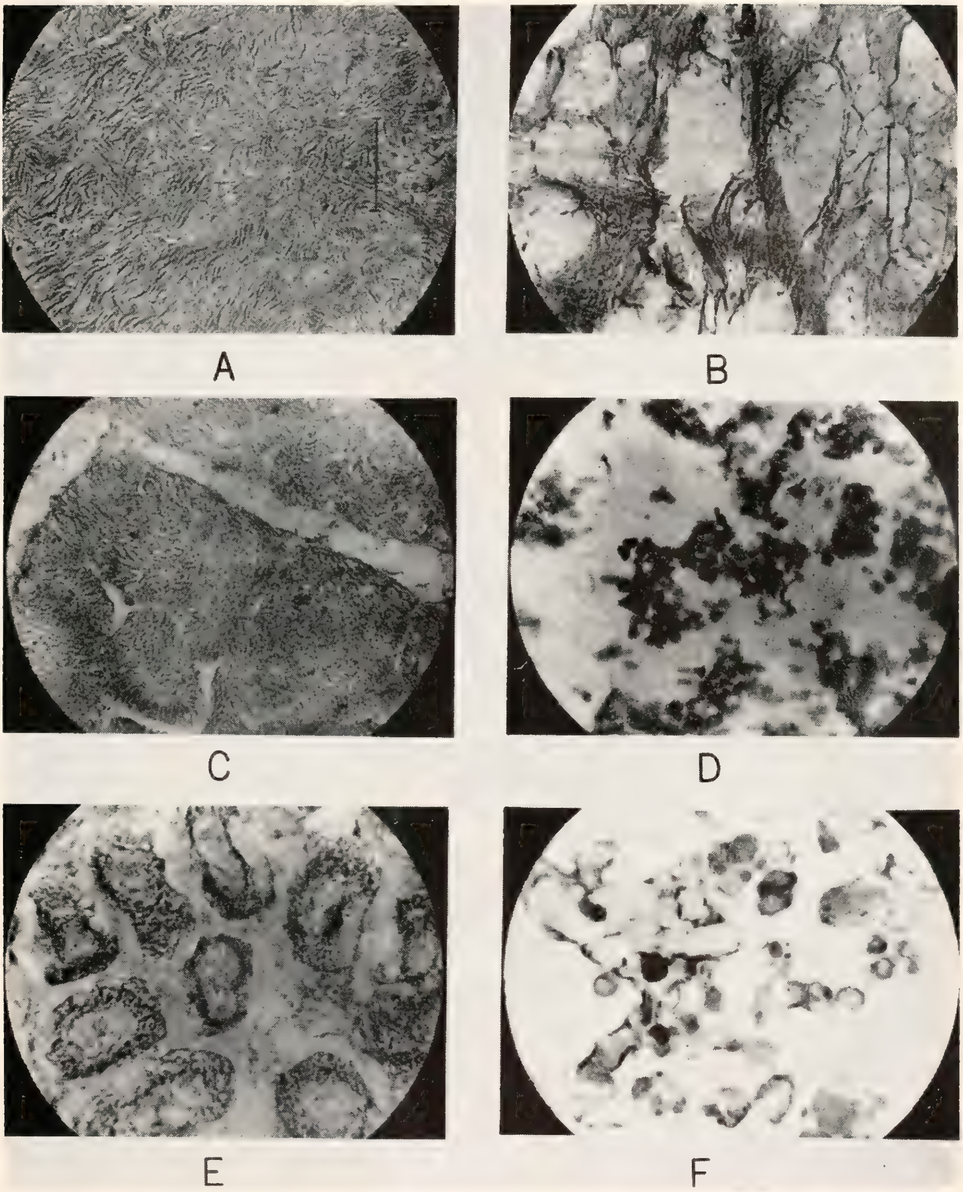

FIGURE 1. Photomicrographs of unfrozen and frozen tissue.

Figure 1, A is the unfrozen foot of the shore snail *Littorina littorea.* The purpose of the randomly arranged muscle fibers is related to the snail's type of locomotion. In the frozen tissue in Figure 1, B, the ice forms in large pockets with a resulting shrinkage and distortion of the cells.

The extreme distortions indicated in the initial results were surprising enough to warrant the following procedure. The frozen muscle slice was cut in two pieces. One was used as the frozen specimen. The other was warmed for less than a minute on the palm of my hand and then hard frozen in the liquid nitrogen. When sectioned it appeared nearly the same as tissue from an unfrozen animal. Figures 1, A and 1, B are actually sections from this run.

A transverse section of the unfrozen adductor muscle in the oyster, *Crassostrea virginicus,* is shown in Figure 1, C. The parallel muscle fibers are viewed end-on. In its frozen counterpart in Figure 1, D, the fibers are clumped into groups to make room for the intervening ice. The prominent elements that resulted are all about the same size. There may be membranes not visible in the unfrozen muscle to account for this regularity. The same regular clumping was seen in the adductor muscle of two mussels, *Modiolus modiolus* and *Mytilus edulis.*

Figure 1, E is of the eggs in the unfrozen ovary of the blue mussel *Mytilus edulis.* When frozen as in Figure 1, F, the detail is much less distinct but the eggs clearly have shrunken during the formation of the large amounts of intercellular ice. Comparable distortions were seen in other tissues from these and other species.

METABOLISM OF FROZEN ANIMALS

Scholander *et al.* (1953) measured respiration at freezing temperatures by following the decrease in oxygen concentration in a closed volume containing the animal. The same method has been used here. Manometric and volumetric techniques can not be used because of the volume change when water turns to ice.

The snails to be used were frozen in 20-ml. syringes in a cold bath. Only those in which the snail froze while fully extended from its shell were used. A short section of tubing on the tip of the syringe extended above the surface of the liquid and was closed with a pinch clamp. A sample of gas could be withdrawn without removing the syringe from the bath. The plunger was free to move up and replace the volume lost in sampling. Allowance was made for the decreased volume in calculating the rate of oxygen removal.

Duplicate oxygen analyses good to 0.02 per cent were made with the half-cc. analyzer of Scholander (1947). Serial samples were plotted against time and the slope was used in computing the oxygen consumption. The concentration was never allowed to go below 18 per cent in any run. Respiration was assumed to be independent of tension over this small range.

After the snails were placed in the cold bath, at least 6 hours were allowed for phase equilibration between ice and water in the tissues. Previous experience (Kanwisher, 1955) had shown that there was no appreciable increase in ice after this length of time. The syringe was then flushed with cold outside air. A series of oxygen determinations showed that such air did not vary appreciably from 20.94 per cent so this was considered the starting concentration. At intervals ranging from 2 to 120 hours samples were withdrawn with a mercury gas sampler.

Volumetric respirometers (Scholander *et al.,* 1952) were used above 0°. One ml. of sea water was included in the vial with the animals. At 0° such values were in good agreement with those made by gas analysis which is specific for oxygen. The often used and rarely proven hypothesis is thus confirmed that the volume decrease is due to oxygen being consumed.

The respiration temperature data from −10 to +30° C. are plotted in Figure 2. Between 0 and +20°, oxygen consumption shows the usual logarithmic increase with a Q_{10} of 2 to 3. Above this respiration decreases, probably due to thermal injury. Below 0° the metabolic activity drops sharply with an apparent Q_{10} of about 50.

At −10°, respiration was so low it took 6 days for the snails to consume a measureable amount of oxygen. Even in this length of time the concentration change was smaller than desired for accurate determination. This may account for the greater spread of values at this low temperature. At −15° the empty syringes gave blank values of one-third the oxygen decrease in those containing snails. This may be due to oxidation of grease used on the syringe plunger. It did not seem that this technique could be trusted on the slower rates to be expected at still lower temperatures.

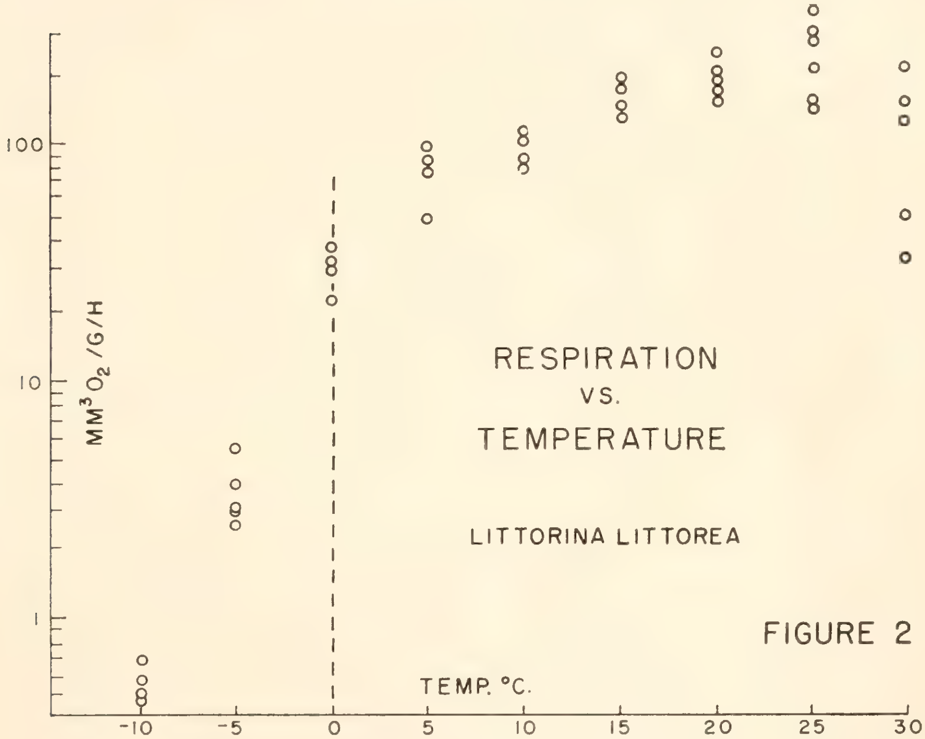

FIGURE 2. Variation of oxygen uptake with temperature.

SALINITY EFFECT ON RESPIRATION

Scholander *et al.* (1953) have given several reasons why the respiratory gas exchange of a frozen animal drops so much more rapidly with temperature than it does above 0° when no ice is present. The ice may act as a diffusion barrier to the gases. The increased viscosity of the body fluids may slow the reaction rates. Finally the increased salinity may directly inhibit the animal's metabolism. No way could be devised to test the first two hypotheses. The respiratory response to increased salinity above 0° can be determined independently of any ice effects.

Higher than normal salinities were made by freezing sea water and using the brine. Dilution with fresh water gave lower than normal salinities. Freshly collected snails were placed in jars containing the different salinities for a minimum of 6 hours before being used. At very high and low values the snails withdrew into their shells. Experience had shown that the operculum blocks respiratory gas exchange so these could not be used.

For the respiration measurements single snails were placed in 20-ml. syringes filled with the desired salinity. The syringes were kept in a constant temperature bath except when sampling. One-ml. samples were removed at convenient intervals and analyzed gasometrically for oxygen by the method of Scholander *et al.* (1955). Since this is a physical extraction of the gases it could be relied on in spite of the water sometimes becoming cloudy with waste products.

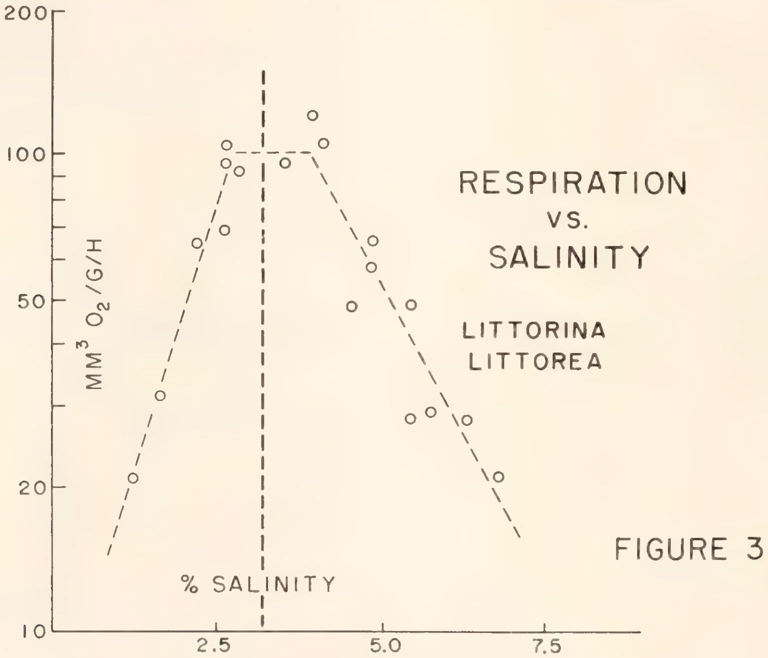

FIGURE 3. Response of oxygen uptake to different salinities.

As in the low temperature gas analysis method several serial readings were used to indicate the rate of oxygen removal by the snails. Low oxygen tensions were avoided by working in the range of 2.5 to 6 mm.³ of oxygen per ml. The curves showed that respiration was independent of tension over this range.

The variations of oxygen consumption with changes in the external salinity are shown in Figure 3. High salinity depresses the respiration of *Littorina littorea*. This is a reversible effect since the rate increases again when the snail is returned to normal salinity. When the snails withdrew into their shells at higher salinities than shown, no oxygen consumption could be detected. They are apparently able to subsist for long periods anaerobically.

Since freezing occurs throughout the animal, the remaining body fluids in all parts of the animal are concentrated. If any effect of external salinity above 0° is to be related to freezing, it must be shown that the animal is not osmotically regulating. Increased salinity could conceivably be effective in only altering the absorption of oxygen at the surface. Tissues of snails from water of different salinities were analyzed for chlorides. By carefully cracking the shell, the animal could be removed whole. Excess water was mopped off and the weight quickly taken. The water was removed by drying for several hours in a 100° oven. The dry weight then gave the total water by difference. The dry tissue was then digested and titrated for the amount of total chlorides present. This amount was considered dispersed in all the animal water in order to calculate the concentration in the live animal. From the resulting normalities at different external salinities it was clear that internal chloride concentration was proportional to that outside the animal. Any result of externally varied salinity can reasonably be viewed as arising from a corresponding change throughout the animal.

Discussion

Chambers and Hale (1932) observed plant and animal cells freezing under the microscope. They found that ice formation inside the cellular membrane always resulted in the death of the cell. The detailed studies of Asahina and his colleagues (1954) have described the freezing process in the blood and isolated organs of insect pre-pupae and in the intact insects themselves. They also found that intra-cellular freezing is lethal to the cell or tissue. Such results are responsible for the general belief that all freezing in animals and plants takes place outside the cells.

The impressive liquid air freezing experiments of nematodes by Luyet and Gehenio (1940) have almost certainly been an instance of ice within cells. Lack of injury here has been attributed to the very rapid rate of freezing. This vitrification does not allow time enough for ice crystals to grow to a size where they can damage protoplasmic structures. It forms the basis of the histology used here. This phenomenon probably has little to do with the normal ecology of these animals in nature.

Shore animals that are exposed to freezing are in shells. This impedes heat transfer and gives ice crystals time to grow. One can readily see them in an opened animal. The tissues of such an animal are similar in texture and appearance to a frozen piece of meat. It is not surprising when one considers that water makes up three-fourths of the bulk of the animal and four-fifths or more of it may be ice. The photomicrographs presented here show the large amount of distortion necessary at the cellular scale to make room for this ice. Yet this can change back to a more or less normal appearing tissue in 30 seconds as shown in Figures 1, A and B. Siminovitch and Briggs (1949) have related frost hardiness in plants to the ability of water to migrate rapidly in and out of the cells. Unfortunately no equivalent test could be devised to apply this to shore animals.

Littorina littorea was found to remain out of its shell and behave normally in salinities of 2 to 7 per cent. It sharply decreased its metabolism in response to a salinity increase. In Figure 3, the data indicate that doubling the salinity above the optimum decreases the oxygen demand to about a third. As the salinity of the body fluids is increased by the freezing out of water, oxygen uptake must drop in the same fashion. From the freezing curves in a previous paper (Kanwisher, 1955) 70 to 80 per cent of the water in this species is frozen at −10°. This would result in a Q_{10} from the salinity of about 10. Above 0° the Q_{10} due to the usual temperature effect on reaction rates is between 2 and 3. Combining these one would expect a Q_{10} in the range of 20 to 30 below 0°. The actually observed one is closer to 50. The effect of ice as a gaseous diffusion barrier and that from the loss of water itself may account for the difference. It is felt that the present data do not warrant a more vigorous interpretation. Similar work with intertidal algae (Kanwisher, 1957) has shown that the drying effect of freezing was chiefly responsible for a similar large decrease in respiration. A three-times increase in salinity had little effect on the oxygen uptake of these plants.

Freezing in shore animals to the extent shown here is a normal occurrence twice daily in the winter with no obvious injury to the animal. This freezing hardiness is probably connected with the ability to stand the internal distortions and high salinities that result. The greatly lowered metabolism may be of adaptive significance in severe locations where shore animals are frozen into the ice for months at a time. As such it could represent a considerable saving in food reserves.

SUMMARY

1. Histology of frozen shore animals has shown large pockets of intercellular ice with consequent shrinkage and distortion of the surrounding cells.

2. The Q_{10} drops precipitously in the region of ice formation and may be as high as 50.

3. High tissue salinity without freezing decreases oxygen uptake. Thus the salinity increase that results from freezing is responsible for a large part of the high Q_{10}.

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CHROMATOGRAPHIC ANALYSES OF AMINO ACIDS IN THE DEVELOPING SLIME MOLD, DICTYOSTELIUM DISCOIDEUM RAPER[1]

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The slime mold, *Dictyostelium discoideum* Raper, is a relatively simple biological system in which to study the processes of differentiation and morphogenesis. From a seemingly homogeneous mass of cells (the aggregation mass), there are eventually formed in the mature sorocarp two basic cell types—the stalk cell and the spore cell. The developmental cycle of *D. discoideum* has been described in detail by Bonner (1944) and Raper (1935, 1940) and will not be repeated here.

In the recent literature, studies have been reported that suggest correlations between nitrogen metabolism and the processes of differentiation and morphogenesis in this slime mold. Gregg, Hackney and Krivanek (1954) detected the evolution of ammonia and described changes in several nitrogenous fractions during the life cycle of this organism. In this same study, they suggested that the cellulose of the mature sorocarp was synthesized at the expense of a protein precursor and pointed out that the major nitrogen changes took place while the spore and stalk cells were being formed, *i.e.*, during the culmination process. In addition, Krivanek and Krivanek (1958), using the histochemical technique devised by Francis (1953), demonstrated the occurrence of amine oxidase activity in various regions of the slime mold undergoing differentiative changes. The simultaneous occurrence of changes in nitrogen metabolism and of differentiative and morphogenetic phenomena prompted the present study.

MATERIALS AND METHODS

The method as outlined by Block, Durrum and Zweig (1955) was used for ascending paper chromatographic determinations of amino acids in the slime mold. Chromatograms, using hydrolyzed and unhydrolyzed tissues, were made of four representative stages of development—migrating pseudoplasmodium, pre-culmination, culmination, and mature sorocarp. In the case of hydrolyzed tissue, individuals in the desired stage of development were isolated and homogenized in 6 N HCl, hydrolyzed for 18 hours, and evaporated over a boiling water bath. The residue was placed in a soda lime desiccator for 48 hours and then taken up in 2 cc. of warm glass-distilled water and filtered. After evaporating the water filtrate, the residue therefrom was taken up in 1 cc. of iso-propanol, the vehicle used in the application of the spot. In the case of the unhydrolyzed tissue, homogenates were made with water and evaporated. The residue was taken up in 1 cc. of iso-propanol and applied to the paper.

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The microhomogenizer described by Gregg, Hackney and Krivanek (1954) was used for the preparation of the tissue homogenates. All homogenization took place at room temperature (22° C.). Depending upon the stage of development to be analyzed, the homogenization procedure lasted from thirty minutes to an hour. All evaporation took place over a boiling water bath with the evaporation lasting no more than five minutes in any case. Rupture of virtually all cells was insured by means of periodic microscopic examination of the homogenate.

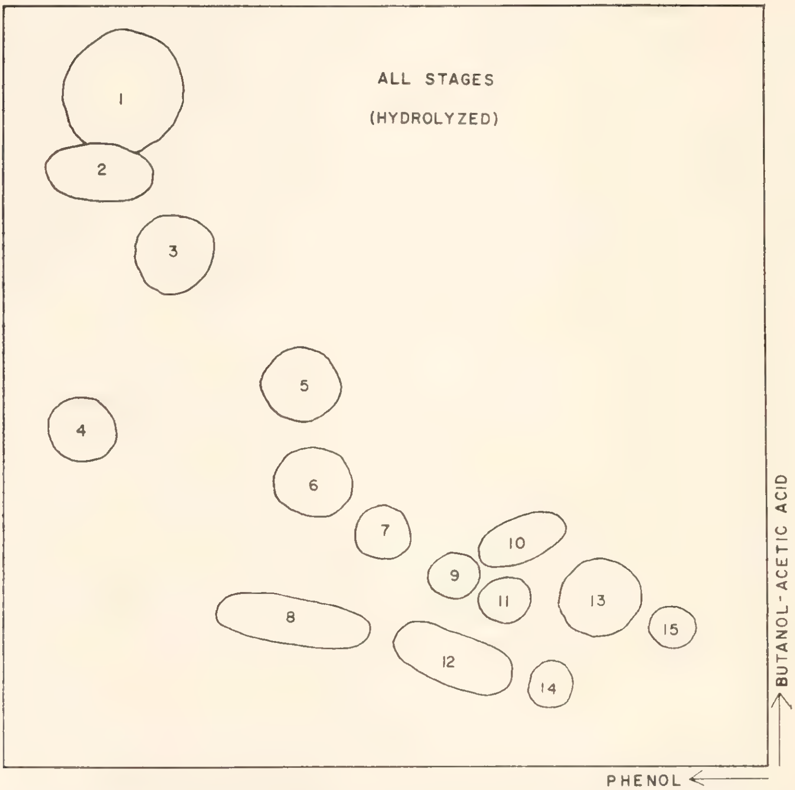


FIGURE 1. Diagram of the results of two-dimensional chromatography on hydrolyzed tissue of *D. discoideum*. Spots are identified as the leucines (1), phenylalanine (2), methionine (3), proline (4), tyrosine (5), alanine (6), threonine (7), histidine (8), glycine (9), glutamic acid (10), serine (11), asparagine (12), unknown (13), cystine (14), and aspartic acid (15).

For both types of analyses, *i.e.*, hydrolyzed and unhydrolyzed, two-dimensional chromatograms were made on Whatman No. 1 filter paper. For the first dimension, n-butanol, acetic acid and water (250, 60, 250 v/v/v) were used as the solvent mixture. For the second dimension, an 80% solution of phenol in water was used as solvent. Development of the spots was accomplished by means of spraying the chromatograms with a solution of 0.3% ninhydrin in 95% ethanol. After spraying, the chromatograms were allowed to dry in complete darkness for 18 hours. No less than 6 and no more than 10 runs were made for each analysis. In the majority of cases, consistent spot patterns were achieved and only 6 runs were made. However, in those few cases where slight inconsistencies in the patterns were evident, additional runs were made to achieve reproducibility.

Identification of the spots was achieved in two ways. Firstly, R_f values were calculated and compared with the R_f values of known amino acids. Secondly, one-dimensional as well as two-dimensional "control" runs were made using solutions of known amino acids, both singly and grouped, and the loci of spots were compared between the control and experimental series.

RESULTS

Hydrolyzed tissue. Results of the chromatographic studies of amino acids in hydrolyzed tissues of *D. discoideum* are shown in Figure 1. With the exception of one spot (no. 13), all spots were identified. The identified spots included the leucines (1), phenylalanine (2), methionine (3), proline (4), tyrosine (5), alanine (6), threonine (7), histidine (8), glycine (9), glutamic acid (10), serine (11), asparagine (12), cystine (14), and aspartic acid (15).

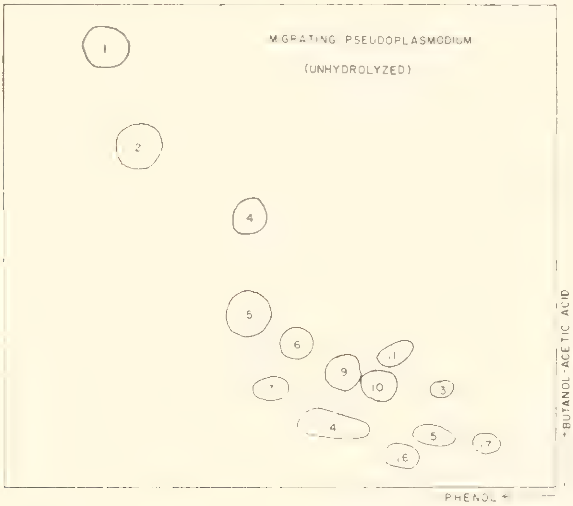
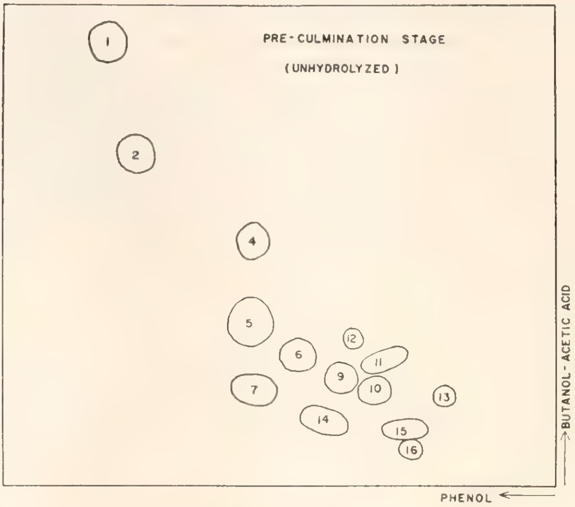


FIGURE 2. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the migrating pseudoplasmodium stage. Identified spots are the leucines (1), methionine (2), tyrosine (4), alanine (5), threonine (6), glycine (9), serine (10), glutamic acid (11), aspartic acid (13), and cystine (15). Spots 7, 14, 16, and 17 are unknowns.

The same spot pattern persisted throughout the four analyzed stages of development. Although no quantitative determinations of the amino acids were made, comparisons of the relative spot intensities afforded some degree of quantification. Glutamic acid presented the most intense color in each stage. Also quite intense, but not to the degree of glutamic acid, were the spots of the leucines, methionine, alanine, threonine, serine, and asparagine. Medium light spots resulted from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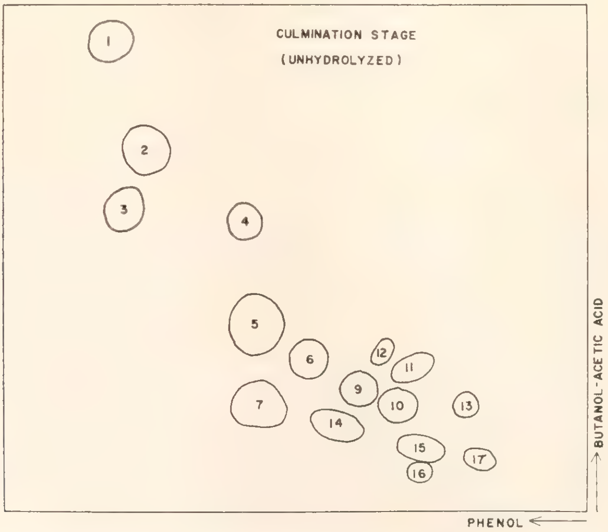
BUTANOL-ACETIC ACID
PHENOL

FIGURE 3. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the pre-culmination stage. Spots as in Figure 2, plus spot 12, an unknown.

phenylalanine, tyrosine, glycine, and histidine. The faintest spots were those of proline, cystine and aspartic acid.

In addition to these well-formed spots, a very faint, vaguely-defined spot was occasionally found in the approximate locus of cysteine. Because of its vagueness


CULMINATION STAGE
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BUTANOL-ACETIC ACID
PHENOL

FIGURE 4. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the culmination stage. Spots as in Figure 2, plus spots 3 and 12, unknowns.

and the failure of our controls to show a clear cysteine spot, we cannot state positively either the presence or absence of cysteine.

Unhydrolyzed tissue. Results of the chromatographic studies of amino acids in unhydrolyzed tissue of *D. discoideum* are shown in Figures 2, 3, 4 and 5. Whereas a consistent spot pattern occurred throughout the developmental cycle in the case of hydrolyzed tissue, considerable variability in the spot patterns occurred between the several stages in the case of unhydrolyzed tissue. A total of 17 spots appeared in all or nearly all of the stages of development. However, only ten were identified. They were the spots of the leucines (1), methionine (2), tyrosine (4), alanine (5), threonine (6), glycine (9), serine (10), glutamic acid (11), aspartic acid (13), and cystine (15). The remaining seven spots—3, 7, 8, 12, 14, 16, and 17—were not identified. Presumably these ninhydrin-positive spots were simple peptides. It is possible that these spots were the products of partial hydrolysis by enzymes derived from the cells. However, in view of the rapidity with which the tissues were prepared, this would seem unlikely. Those spots which were evident in all stages of development were 1, 2, 5, 6, 7, 9, 10, 11, 13, 14, 15, and 16. Although spot 12, an unknown, did not appear in the migrating pseudoplasmodium, it did appear in the succeeding three stages of development. Spot 3, also unknown, appeared only in the culmination and mature sorocarp stages, while spot 8, a third unknown, appeared only in the mature sorocarp stage. Spot 4, identified as tyrosine, was present in all stages except the mature sorocarp, and spot 17 appeared erratically—being present in all but the pre-culmination stage. As in the case of hydrolyzed tissue, cysteine could not be definitely ascertained as either being present or absent.

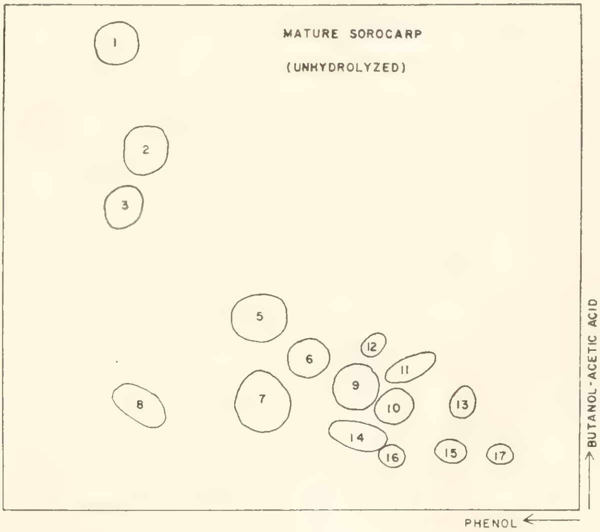

FIGURE 5. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the mature sorocarp stage. Spots as in Figure 2, plus spots 3, 8, and 12, unknowns.

DISCUSSION

In their quantitative studies of the nitrogen metabolism in the slime mold, *D. discoideum,* Gregg, Hackney and Krivanek (1954) demonstrated a decrease in the total nitrogen/dry weight during the transition from the migrating pseudoplasmodium to the mature sorocarp. They attributed this decrease to a decrease in the total extractable protein nitrogen and total unextractable nitrogen components of the slime mold. In addition, they found that ammonia was being given off by the slime mold during its life cycle. In a subsequent study, Gregg and Bronsweig (1956) found a steady increase in the total amount of reducing substances (presumably carbohydrates) as the life cycle progressed. On the basis of these data, it was suggested that the protein fraction of the slime mold served as a precursor for the carbohydrate of the mature sorocarp. However, no indication was made of the possible pathway(s) involved in this conversion. The present study may be suggestive in this respect.

Glutamic acid invariably presented the most intense spot of any of the determined amino acids. This was evident in both hydrolyzed and unhydrolyzed tissues. The deamination of glutamic acid to α-ketoglutaric acid with the corresponding release of ammonia is known. Because of the reversibility of this reaction, it is considered to be one of the prime mechanisms responsible for the interconversion of ammonia and α-amino group nitrogen. The reaction is catalyzed by glutamic acid dehydrogenase, requiring either DPN or TPN as a coenzyme (Meister, 1957). The importance of this reaction, as it relates to *D. discoideum,* lies in the fact that not only has glutamic acid been detected to a high degree in the slime mold, but, also, the liberation of ammonia during the life cycle suggests such a deamination reaction. Further, Krivanek and Krivanek (1958) demonstrated non-specific dehydrogenase activity in the pre-stalk area of the pre-culmination and culmination stages—stages in which the future sorophore sheath (consisting primarily of polysaccharides) is being secreted by the stalk cells as they move apically to become eventually enclosed within the sorophore sheath. This non-specific dehydrogenase activity could logically be attributed to glutamic acid dehydrogenase. By virtue of the relationship between glutamic acid, α-ketoglutarate, areas of dehydrogenase activity, and sites of carbohydrate secretion, there thus can be postulated this link between carbohydrate metabolism and protein metabolism in the slime mold.

The glutamic acid-ketoglutarate relationship, if actually operative in the slime mold, need not be the only link between carbohydrate metabolism and protein metabolism. Aspartic acid, also demonstrated in hydrolyzed and unhydrolyzed tissues of the slime mold, can be deaminated to fumarate, another intermediate in the citric acid cycle (Meister, 1957), thus creating a second possible link between the two types of metabolism. Further, there is the possibility that alanine can undergo deamination forming the Krebs cycle intermediate—pyruvate—as has been suggested by Meister (1957), and serine, as well as cysteine, can undergo the same process yielding ammonia and pyruvate.

The suggested relationships already discussed do not preclude the possibility of other mechanisms relating carbohydrate metabolism to protein metabolism, such as decarboxylation and transamination. There is as yet, however, no evidence to indicate the presence of these mechanisms in the slime mold.

Several of the amino acids of the hydrolyzed tissues appear as well in unhydrolyzed tissue. Consequently, it is not possible to determine whether these amino acids occur as free amino acids only, or also as bound amino acids. However, four amino acids appear only in the hydrolyzed tissue (phenylalanine, proline, histidine, and asparagine). They are considered therefore to exist only in the bound form. The significance of these amino acids with respect to the differentiative process in *Dictyostelium* is at present not apparent.

Studies have recently been initiated to test the validity of the above postulates. These correlative studies will embrace the use of the analogs of the amino acids shown to be present in *D. discoideum.*

SUMMARY

1. The amino acids in hydrolyzed and unhydrolyzed tissue of the slime mold, *Dictyostelium discoideum* Raper, have been determined by means of two-dimensional ascending paper chromatography. Analyses were made on four stages of development—migrating pseudoplasmodium, pre-culmination, culmination, and mature sorocarp.

2. Unhydrolyzed tissue contained the leucines, methionine, tyrosine, alanine, threonine, glycine, serine, glutamic acid, aspartic acid, cystine, and seven unidentified spots, presumably simple peptides. Not all these spots were present in all tested stages.

3. Hydrolyzed tissue contained in addition to the amino acids identified above, phenylalanine, proline, histidine, asparagine, and one unknown spot. All tested stages were identical.

4. The postulate is presented that glutamic acid (and possibly also to a lesser degree aspartic acid, alanine, serine, and cysteine) through deamination may enter the Krebs cycle and form a link between protein and carbohydrate metabolism, the change in balance between protein and carbohydrate being one of the most prominent features of differentiation in this organism.

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SOME ASPECTS OF OSMOREGULATION IN TWO SPECIES OF SPHAEROMID ISOPOD CRUSTACEA

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The internal effects of osmoregulation were studied in two euryhaline species of isopod crustaceans, *Gnorimophaeroma oregonensis* (Dana) and *Sphaeroma pentodon* Richardson. Although a large literature exists on the subject of osmoregulation in Crustacea, only a little of it concerns isopods. Therefore, the present study was undertaken to gain more information in this relatively unexplored area.

Bogucki (1932) studied the ionic composition of the body fluid in *Mesidotea entomon,* which according to Ekman (1953) is an inhabitant of the Baltic and Arctic Seas and several fresh-water lakes in the land area of the northwest Pacific, Siberia, and northern Europe. Bogucki found the body fluid concentration to be hypertonic to the medium in lower salinities, becoming isotonic as the medium approached sea water. Lockwood and Croghan (1957), studying the brackish- and fresh-water races of the same species, found the osmotic behavior to be similar in both races, except that the brackish-water animals could not survive in fresh water. They concluded that the fresh-water race has developed a more effective osmoregulatory mechanism that enables it to maintain the high haemolymph concentrations of the brackish-water race in fresh water. Bateman (1933) found that *Ligia oceanica* maintained its body fluid hypertonic to a medium of about 80 per cent sea water, but swelled and died in 50 per cent sea water. However, Parry (1953), working with *Ligia exotica,* found that in well-aerated sea water, specimens of the species could survive 17 to 30 days in salinities ranging from 50 to 125 per cent sea water. In very concentrated media ($\Delta = 3.46°$ C.), the body fluid was maintained hypotonic to the medium. Menzies (1954), in addition to splitting *Gnorimosphaeroma oregonensis* into two subspecies, *lutea* and *oregonensis,* performed preliminary experiments to test the ability of the two subspecies to survive in various salinities. Specimens of *G. o. oregonensis* taken from 25 per cent sea water and placed in tap water were all dead after one day. Specimens of *G. o. lutea* taken from 1.6 per cent sea water and placed in tap water died slowly over a period of three days. Menzies concluded that *G. o. oregonensis* is probably restricted to sea water, but he was puzzled as to why *G. o. lutea* could survive in sea water, but not in tap water (salts equivalent to 0.3% sea water), which was not very much less saline than the normal habitat water (1.6% sea water). He postulated that *G. o. lutea* required a slight concentration of salts, greater than the tap water used, or that there were toxins present in that medium.

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MATERIALS AND METHODS

Experimental animals

Gnorimosphaeroma oregonensis is widely distributed over the west coast of North America from Alaska to central California (Menzies, 1954). It also occurs in Hawaii (M. A. Miller, unpublished report). It may be collected intertidally in bays, in estuarine conditions and occasionally populations of the species are found in fresh-water creeks and ponds. Because of its ability to inhabit such a wide ecological range, it was considered to be a suitable experimental type for the further elucidation of osmoregulatory adaptations which enable a marine animal to live in brackish and fresh water.

The following designations will be used for the three habitat groups of *Gnorimosphaeroma oregonensis.* Animals taken from fresh water will be called *G. oregonensis* (FW); those taken from estuarine populations will be called *G. oregonensis* (EF = estuarine form), and those animals taken from intertidal bay populations will be designated *G. oregonensis* (BF = bay form). *G. oregonensis* (FW and EF) equal the subspecies *G. o. lutea* of Menzies. *G. oregonensis* (BF) equals the subspecies *G. o. oregonensis* of Menzies.

Sphaeroma pentodon is known only from San Francisco Bay (Richardson, 1905), Tomales Bay (new locality), and Bolinas Lagoon (new locality), California. It lives intertidally in burrows, which it constructs in mud, wooden logs and pilings, and sandstone. The salinity of the habitats from which it was collected ranged from about 11 per cent sea water to about 96 per cent sea water. *S. pentodon* was included in the study because it is related to *Gnorimosphaeroma oregonensis,* and its range overlaps that of the latter species in parts of its distribution.

Methods

Four principal types of studies were made: (1) Changes in the total osmotic pressure of the body fluid after three, 24, and 48 hours exposure to the experimental salinities were made in order to determine the relative degree and pattern of osmoregulation exhibited by the animals. (2) The animals were weighed before and after exposure to the experimental salinities for 24 hours in order to detect possible changes in weight indicating water gain or loss. (3) Survival tests were run to determine the length of time the experimental animals could live in the experimental salinities. (4) Field checks were made by measuring changes in the body fluid of all but *Gnorimosphaeroma oregonensis* (FW) during a portion of a tidal cycle.

The laboratory experiments were conducted at 16° C., a temperature to which all forms were accustomed. The animals were placed in 60 per cent sea water for 24 hours prior to the start of the experiments. The 60 per cent sea water permitted a common starting salinity for all experimental series, facilitating comparisons.

Experimental salinities

The experimental salinities used in this study were 125, 100, 75, 50, and 25 per cent sea water, and fresh water (salts equivalent to 0.25% sea water by chloride determination). The 100 per cent sea water (salinity = 34.44‰) was collected off the Marin County coast away from the influence of fresh-water streams. The 25, 50, and 75 per cent sea water solutions were made by diluting normal sea water with distilled water. The 125 per cent sea water solution was prepared by boiling normal sea water, taking care not to precipitate salts. The pH was checked before and after boiling to ascertain that any loss of carbon dioxide was regained by exposure to air. The fresh water was soft creek water collected at Pilarcitos Creek, San Mateo County, California.

Salinity determinations

Salinity determinations on sea water concentrations greater than 25 per cent sea water were made by a short method described by Welsh and Smith (1953). The salinity of sea water diluted to less than 25 per cent normal sea water and fresh water was determined by the standard silver nitrate titration method using the Knudsen Tables (1901).

Melting point determinations

A method devised by Gross (1954) was used for determining the melting point of body fluids. From repeated runs on standard samples, it was found that the concentration of the body fluids could be obtained within an error of about two per cent sea water (0.04° C.).

Body fluid samples (*ca.* 1–2 mm^3.) were collected into prepared melting point capillaries (*ca.* 1 mm. ID × 3 cm. length) which were previously marked with a coded series of dots corresponding to the experimental salinities to which the animals had been exposed. Collection of the body fluid was facilitated by the use of a hand control. After collection, both ends of the capillary were sealed with petroleum jelly and the sample quick-frozen on dry ice.

Survival tests

The ability of the experimental animals to survive for extended periods of time in the experimental salinities was tested as follows: Seventy animals of each experimental group were placed, ten each, in six jars containing the experimental salinities, and one jar containing filtered habitat water. The jars were checked daily for 21 days, and the number of survivors recorded.

Field tests

Changes in the body fluid concentration of *Gnorimosphaeroma oregonensis* (EF), *G. oregonensis* (BF), and *Sphaeroma pentodon* during a $7\frac{1}{2}$-hour period from low to high tide in the field were measured as follows: In the case of *G. oregonensis* (EF), which remains immersed in water during low tide, five body fluid samples and one sea water sample were taken at $1\frac{1}{4}$-hour intervals. In the case of *G. oregonensis* (BF) and *S. pentodon,* which remain out of the water during low tide, five body fluid samples and five samples of water around the pleopods were collected. The body fluid and pleopod water samples were frozen on dry ice and returned to Davis for determination.

RESULTS

The term "gradient" will be used in the following pages to indicate the difference in concentration (expressed in percentage sea water) between the body fluid and the medium.

Melting point determination of body fluid concentrations

The results of melting point determination of body fluid concentrations are shown in Figure 1. In general, changes in the body fluid concentrations seemed to be rapid—the major changes occurred within the first three hours of exposure to the experimental salinities.

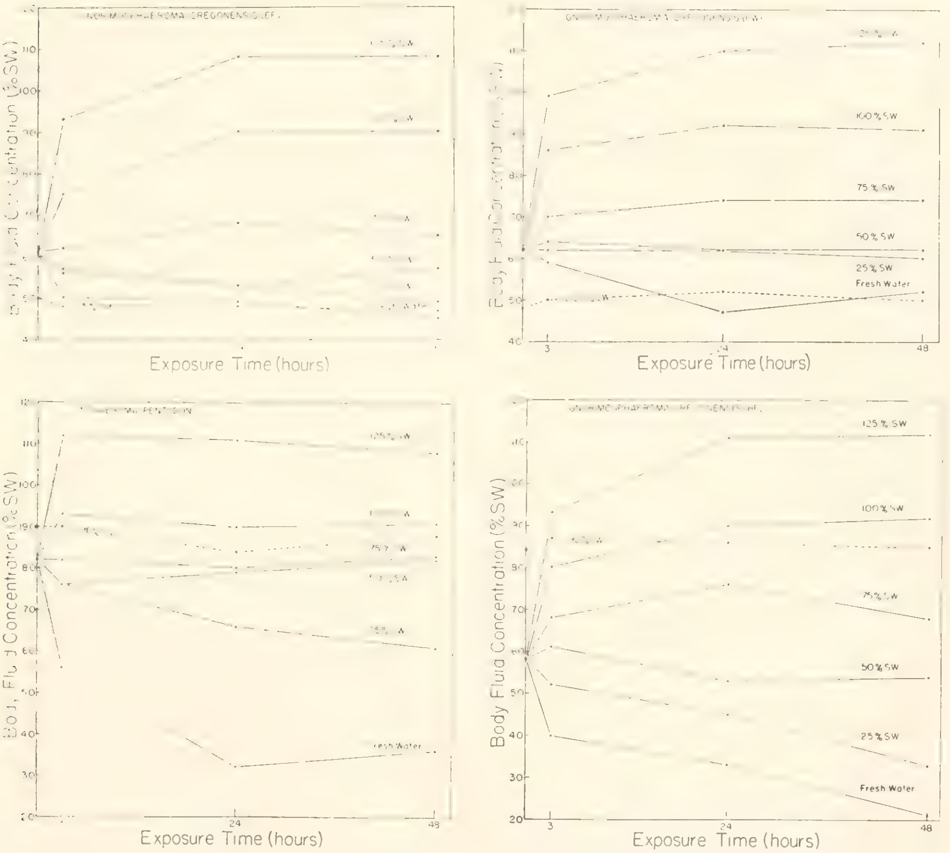

FIGURE 1. Body fluid concentration changes with time in the experimental salinities. The dotted line represents the body fluid concentration changes of animals kept in habitat salinities (controls) indicated.

After 48 hours' exposure, the fresh-water and estuarine forms of *Gnorimosphaeroma oregonensis* maintained their body fluids hypertonic to the medium in 50 per cent sea water and less, and hypotonic in 75 per cent sea water and above. However, in 75 per cent sea water after 24 hours' exposure, the body fluid concentration values of *G. oregonensis* (FW) were quite variable, ranging between hypotonicity and hypertonicity. Possibly that salinity is close to the medium concentration where the "switch" from hyper- to hypo-osmotic regulation occurs. *G. oregonensis* (BF) maintained its body fluid hypotonic to the medium in 75 per cent sea water and above, and hypertonic to 50 and 25 per cent sea water. Apparently, there was no maintenance of the body fluid concentration in fresh water. In that medium, the body fluid concentration steadily dropped, and after 48 hours, all of the animals were dead.

Comparing the above results with those of Menzies (1954) above it can be seen that in both studies, *Gnorimosphaeroma oregonensis* (BF) (= *G. o. oregonensis* of Menzies) could not survive in fresh water. However, in Menzies' study, *G. oregonensis* (EF) (= *G. o. lutea* of Menzies) were not surviving after three days in tap water, while in the present study, that form lived for several days in fresh water. It is possible that the tap water used by Menzies (unchlorinated well water) contained some unknown toxic substance or had an imbalance of ions. Its ion analysis is as follows: HCO_3, 0.241‰; SO_4, 0.037‰; Cl, 0.029‰; Ca, 0.011‰; Mg, 0.020‰; and Na, 0.078‰.

After 48 hours, *Sphaeroma pentodon* maintained its body fluid hypotonic to the medium in 100 and 125 per cent sea water and hypertonic in the lower salinities. It is interesting to note that *S. pentodon* and *Gnorimosphaeroma oregonensis* (BF) have extremely wide viability limits in terms of the concentration and dilution of their body fluids—surviving within a concentration range (of their body fluids) of over 70 per cent sea water!

Weight changes in the experimental media

No weight changes were detected in any of the experimental animal groups, except *Gnorimosphaeroma oregonensis* (BF) in fresh water. In that salinity, the majority of the animals were very close to death at the end of the 24-hour period, and the weight changes were considered to be subnormal. Those animals that were still active at the end of the 24 hours did not lose weight. It was possible to weigh the animals within an average error of one per cent of their body weight.

Survival tests

The survival experiment was terminated after 21 days. At termination, the estuarine and fresh-water forms of *Gnorimosphaeroma oregonensis* were surviving in all salinities. *G. oregonensis* (BF) was surviving in all salinities except fresh water, where the LD_{50} value (average survival time) was less than two days. *Sphaeroma pentodon* was surviving in all experimental salinities, except fresh water, where the LD_{50} value was 11 days. No unusual mortality was noted among the controls.

Field tests

The results of the field test of body fluid concentration changes during a tidal cycle showed that no significant changes in concentration of the body fluid or water surrounding the pleopods were detected in *Gnorimosphaeroma oregonensis* (BF) or *Sphaeroma pentodon.* In *G. oregonensis* (EF), however, changes were rather characteristic. Starting at low tide, when the animals were exposed to fresh water, the body fluid concentration was 50 per cent sea water. This concentration did not change until over five hours later, when the salinity of the habitat had reached 42 per cent sea water, at which time the body fluid concentration was 58 per cent sea water. Then, by the time of the extreme high tide, 1¼ hours later, the body fluid concentration had changed again to 70 per cent sea water, while the medium concentration had changed to 65 per cent sea water.

Discussion

Comparative osmoregulatory abilities

Figure 2 shows the 48-hour body fluid concentrations of the experimental animals in the experimental media. It was assumed that all major changes in body fluid concentration had occurred by 48 hours. In hypotonic media, *Sphaeroma pentodon* appears to be a strong regulator, at least in 50 and 75 per cent sea water. There is no apparent reason for the animals to maintain such high body fluid concentration in those salinities when they can live, at least for several days, in fresh water and 25 per cent sea water with (presumably) much lower body fluid concentrations. *Gnorimosphaeroma oregonensis* (BF) has only limited regulation in all media and appears to be the greatest conformer of the group, maintaining a relatively small gradient between its body fluid and the medium in all salinities. *G. oregonensis* (EF) and *G. oregonensis* (FW) are the most able regulators in terms of the ability to maintain their body fluid concentrations relatively constant in hypotonic media. The body fluid concentration differences between the two forms seen in fresh water, 25 per cent sea water, and 50 per cent sea water, are statistically significant ($t = 6.15$, 3.87, and 12.3, respectively, with 11, 10, and 9 degrees of freedom). The ability of *G. oregonensis* (FW) to maintain its body fluid more concentrated in the hypotonic media perhaps represents the major osmoregulatory difference between the two forms. The estuarine form is intermediate between the bay and fresh-water forms in osmoregulatory ability.

Comparing the osmoregulatory abilities of the isopods in this study with those of other crustaceans, a similarity can be seen to species inhabiting similar salinity ranges. From the results of Lockwood and Croghan (1957), it appears that *Mesidotea entomon* is similar in its osmotic regulation to *Gnorimosphaeroma oregonensis.* The former species consists of two "races" which have adapted to brackish- and fresh-water. As in *G. oregonensis* (FW), the fresh-water *M. entomon* is able to live in salinities up to normal sea water. However, unlike *G. oregonensis* (EF), the brackish-water "race" of *M. entomon* cannot live in fresh water. The brackish-water *M. entomon* is thus closer to *G. oregonensis* (BF) and *Sphaeroma pentodon* in its osmoregulatory abilities. However, *M. entomon* does not show the high degree of hypo-osmotic regulation seen in the isopods in the present study. Beadle and Cragg (1940) reported a difference in the ability to retain chloride between the brackish- and fresh-water forms of the amphipod, *Gammarus duebeni,* when placed in distilled water. The fresh-water form retained sufficient chloride to survive for several days in distilled water, whereas the brackish-

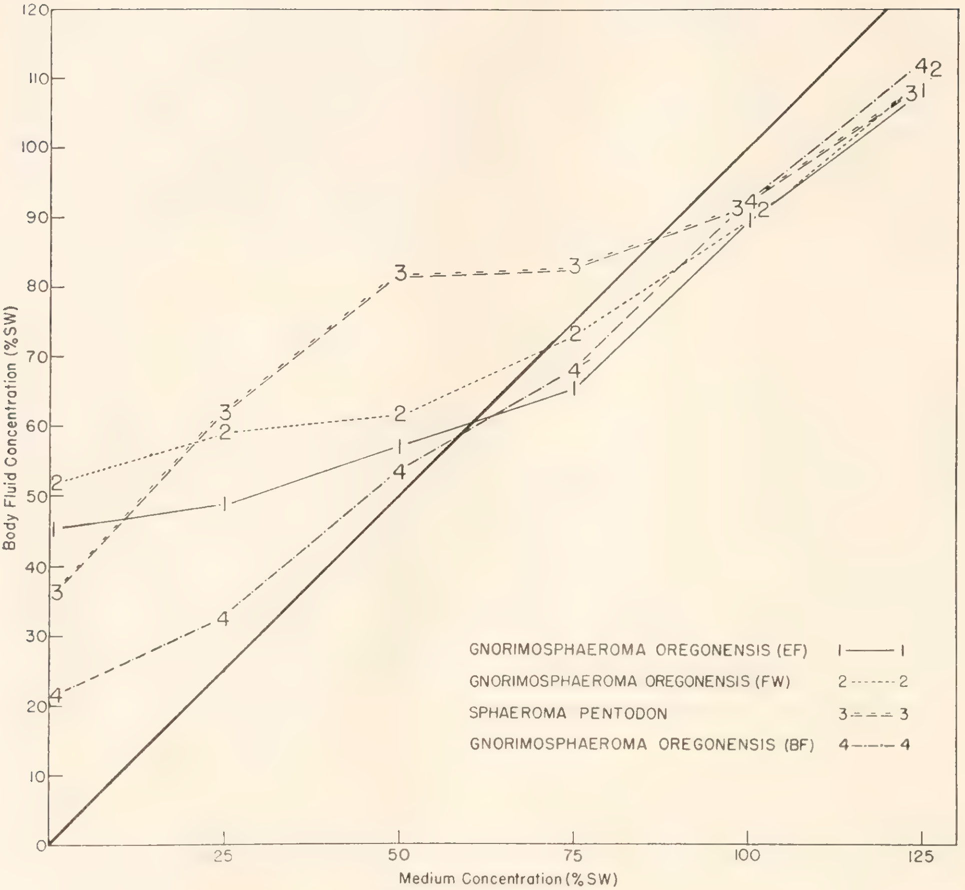

FIGURE 2. Relation of the body fluid concentration to the medium concentration of animals exposed for 48 hours to the experimental salinities.

water form lost chloride and died rapidly in that medium. It appears that the osmoregulatory abilities of the isopods in this study are intermediate between those of one group of crustaceans which can hyper-regulate in dilute sea water, but become isosmotic, or nearly so, in salinities approaching normal sea water (*e.g., Carcinus maenas,* Schlieper, 1929; *Hemigrapsus oregonensis* and *H. nudus,* Jones, 1941) and a second group of crustaceans, which hyper-regulate in dilute sea water and hypo-regulate in salinities approaching normal sea water (*e.g., Heloecius cordiformis,* Edmonds, 1935; *Uca crenulata* and *Pachygrapsus crassipes,* Jones, 1941; *Palaemonetes varians,* Panikkar, 1941; and *Palaemon serratus,* Parry, 1954). All but the last two members of the latter group are primarily semi-terrestrial, which has led Prosser *et al.* (1950a, 1955) to suggest that hypo-osmotic regulation may be associated with the semi-terrestrial habit. The isopods in this study are able to survive for extended periods out of water, but they cannot be classified as semi-terrestrial.

The mechanism of osmoregulation

Although there is little direct evidence elucidating the actual mechanisms of osmotic regulation of the body fluid of the experimental animals, it is possible to make certain hypotheses concerning that phenomenon based on data obtained in the present study and in studies (unpublished) which were made prior to the present study.

a. *Evidence for water movement*

There were no detectable weight changes in the experiments conducted at 16° C., which indicates that there was no net gain or loss of water from the experimental animals' bodies. It is probable that the maintenance of a zero net water flux (that is, no imbalance of the water gain/loss ratio) is dependent upon the ability of the animal to maintain its metabolic rate at a normal level. Duplicate experiments done at 5° C. (see Riegel, 1958) resulted in weight gains by the experimental animals in the dilute salinities and weight losses in the more concentrated salinities. These results may be interpreted as being due to an interference by the low temperature with the normal metabolism of the animals.

b. *Evidence for salt movement*

Since there were no weight changes in the experiments conducted at 16° C., it must be assumed that body fluid concentration changes were due to salt movement. In dilute media (fresh water to 50 per cent sea water), the salt concentration of the body fluid was actively maintained against a gradient. In more concentrated media (75 to 125 per cent sea water), salts were prevented from entering the body (or were eliminated as fast as they came in), since after the initial concentration of the body fluids (generally by 24 hours) the body fluid was maintained hypotonic to the medium. This mechanism could possibly involve, at least in part, an arrest of the mechanism for active salt absorption.

Except for *Gnorimosphaeroma oregonensis* (FW) there was a rapid loss of salts (within three hours) in the more dilute salinities. Whether this loss was due to an active elimination of salts by the animal, thus reducing the concentration gradient between their body fluids and the medium, or a passive loss from the body is not known. There is some evidence suggesting an active elimination of salts in the more dilute salinities, shown especially by *G. oregonensis* (EF) and *Sphaeroma pentodon* after three hours' exposure to fresh water. In those two forms, the body fluid concentrations dropped more rapidly at 16° C. than at 5° C. (see Riegel, 1958).

Whatever the mechanism for the maintenance of the body fluid concentrations in lower salinities, low temperatures interfere with the metabolism of the animals, causing variations in osmoregulation not seen at the higher temperature. In all cases, except *Gnorimosphaeroma oregonensis* (BF) in fresh water, the animals were able to maintain their body fluid concentrations within viable limits after 48 hours' exposure at 16° C. But at 5° C., *G. oregonensis* (BF) was dead after 24 hours' exposure to fresh water and 48 hours' exposure to 25 per cent sea water, and *Sphaeroma pentodon* died after 48 hours' exposure to fresh water. Further, the body fluid concentration of *G. oregonensis* (FW) and *G. oregonensis* (EF) dropped to subnormal values in fresh water at the lower temperature, but remained within normal limits at the higher temperature.

Wikgren (1953) studied the effect of low temperature on various poikilothermous animals (a crayfish, a lamprey, and a bony fish) and concluded that low temperatures have their chief effect in interfering with the ion-absorbing mechanism of the animals. In the lamprey, urine production was decreased by low temperature, which may have resulted in a weight gain, although Wikgren did not indicate that such was the case. David (1925) performed experiments on the living kidney of the frog, which indicated that that organ's urine diluting and concentrating activity was not affected by temperature. However, Wikgren (1953) recalculated David's data and stated that the diluting capacity of the frog's kidney was reduced by low temperature. Thus, evidence may be inferred from the review by Wikgren (1953) that low temperature adversely affects the ability of cold-blooded animals (at least, cold-blooded vertebrates) to rid the body of water.

The changes in body fluid concentration seen in the present study at 16° C. were undoubtedly due to salt movement. Since there were demonstrated water losses and gains at 5° C., the question arose as to whether the body fluid concentration changes which occurred at that low temperature were due entirely to water movement or were partly due to salt movement.

Because the usual procedures for determining body fluid volume were hardly applicable to animals of such small size as used in this study, that component was estimated in the following manner. Ten animals of each experimental group were weighed, and all the body fluid removed from their bodies that could be collected into capillaries of 1-mm. bore. The animals were then re-weighed. Average collectable body fluid weights as a percentage of total body weight were 9.5, 9.7, 11.1, and 6. 8, respectively, for *Gnorimosphaeroma oregonensis* (FW), *G. oregonensis* (EF), *G. oregonensis* (BF), and *Sphaeroma pentodon*. These values established the minimum possible weight of the body fluid. Ten animals of each experimental group were weighed and dried to constant weight in a calcium chloride desiccator. The average values for total body water as a percentage of the total body weight were 56.5, 55.6, 56.4, and 53.8, respectively, for *G. oregonensis* (FW), *G. oregonensis* (EF), *G. oregonensis* (BF), and *S. pentodon*. These values established the maximum possible weight of the body fluid as a percentage of the total body weight.

Table I compares the calculated and actual dilution and concentration of the body fluids in fresh water and 125 per cent sea water [using a 40-milligram specimen of *Gnorimosphaeroma oregonensis* (EF) as an example] based on estimates of the body fluid weight ranging from ten to 50 per cent of the total body weight. A sample calculation follows: Referring to Table I, it can be seen that a 40-milligram animal, with a body fluid concentration of 50 per cent sea water (column 5), when placed in fresh water would gain 11.3 per cent of its body weight (column 3) after 24 hours. If the weight gain is due entirely to water entry into the body, the incoming water would dilute the body fluids by a factor, *X*, given by the relation: $X = \frac{\mathrm{wt}_0\ (=\text{original body fluid weight})}{\mathrm{wt}_{24}\ (=\text{24-hour body fluid weight})}$. If the body fluid comprises 50 per cent of the total body weight (column 1), its dilution by the gain of 4.5 milligrams of water (column 4) would result in a body fluid concentration of $X \cdot 50$ $\left(= \frac{20}{24.5} \cdot 50\right)$, or 40.8 per cent sea water (column 6).

When a 40-milligram animal whose initial body fluid concentration is 50 per cent sea water is placed in 125 per cent sea water, if the body fluid comprises 50 per cent of the total weight, the body fluid would be concentrated by the factor $X \left(= \frac{20}{17.3}\right)$. Thus the body fluid will be concentrated to 57.8 per cent sea water (column 10).

TABLE I

Comparison of actual and calculated concentration and dilution of the body fluids (BF) at 5° C. based on several estimates of the body fluid weight (BF Wt.) as a percentage of total body weight (BW) and assuming the concentration and dilution to be due entirely to water movement*

1 Est. BF Wt. (% BW)	2 Est. BF Wt. (mg.)	3 % BW gain FW	4 BF Wt. after 24 hrs. FW	5 Start. BF conc. (% SW)	6 Calc. BF conc. FW	7 Actual BF conc. 24 hrs. FW	8 % BW loss 125% SW	9 BF Wt. 24 hrs. 125% SW	10 Calc. BF conc. 24 hrs. in 125% SW	11 Actual BF conc. 24 hrs. in 125% SW
50	20	11.3	24.5	50	40.8	42	6.7	17.3	57.8	112
40	16	11.3	20.5	50	39.0	42	6.7	13.3	60.2	112
30	12	11.3	16.5	50	36.4	42	6.7	9.3	64.5	112
20	8	11.3	12.5	50	32.0	42	6.7	5.3	75.4	112
18	7.2	11.3	11.7	50	30.8	42	6.7	4.5	80.0	112
16	6.4	11.3	10.9	50	29.4	42	6.7	3.7	86.5	112
14	5.6	11.3	10.1	50	27.7	42	6.7	2.9	96.6	112
13	5.2	11.3	9.7	50	26.8	42	6.7	2.5	104.0	112
12	4.8	11.3	9.3	50	25.8	42	6.7	2.1	114.3	112
11	4.4	11.3	8.9	50	24.7	42	6.7	1.7	129.4	112
10	4.0	11.3	8.5	50	23.5	42	6.7	1.3	153.8	112

* A 40-mg. specimen of *Gnorimosphaeroma oregonensis* (EF) was used as an example.

From Table I it can be seen that the calculated body fluid concentrations and dilutions in 125 per cent sea water and fresh water, based on estimates of the body fluid weight percentage, do not completely match the actual results. If the estimated body fluid weight of 50 per cent total weight is correct, the calculated dilution in fresh water is close to the actual value. However, the calculated concentration in 125 per cent sea water is much lower than the actual value. If the estimated body fluid weight of 12 per cent total body weight is correct, the calculated body fluid concentration in 125 per cent sea water is close to the actual value, but the calculated body fluid concentration in fresh water is much lower than the actual value. Therefore, it is likely that the actual body fluid weight lies somewhere between 10 and 50 per cent of the total body weight. If a reasonable estimate of 20 to 30 per cent is close to the actual value for the body fluid component of the total body weight, it appears that the actual body fluid concentrations in fresh water and 125 per cent sea water at 5° C. are not due entirely to water movement. That is, it is probable that there is a retention or reabsorption of salts in fresh water and an absorption of salts in 125 per cent sea water.

These results are in general agreement with those of Hukuda (1932) who compared the theoretical and actual change in weight with the observed change in osmotic pressure of the blood in *Portunas puber* when that marine animal was immersed in ⅔ normal sea water. Gross (1957) found in *Emerita analoga* that a weight change of less than two per cent of the body weight resulted in a body fluid concentration change equivalent to 25 per cent sea water. Based on the assumption that osmotically active water comprised 40 per cent of the body weight, he calculated that the weight change, if due entirely to water movement, would have changed the body fluid concentration by less than six per cent.

The estimate of 20 to 30 per cent as the haemolymph component of the body weight in *Gnorimosphaeroma oregonensis* (EF) only partially agrees with similar estimates of that value in other crustaceans. A body fluid value of 50 per cent of body weight was assumed by Lockwood and Croghan (1957) for *Mesidotea entomon.* Similarly, a body fluid of ⅓ body weight was assumed for *Palaemonetes antennarius* by Parry (1957). Gross (1957) made actual calculations of the "solute space" in *Pachygrapsus crassipes* and *Emerita analoga* which were, respectively, 56 and 40 per cent of body weight. However, solute space would be expected to be greater than the body fluid volume and less than the total body water. Approximate measurements of blood volume of various crustaceans have been made using sodium thiocyanate. Nagel (1934) found a blood volume of 37 per cent of body weight in *Carcinus maenas.* Krogh (1939) measured a blood volume of 33 per cent of body weight in *Eriocheir sinensis.* Prosser and Weinstein (1950) measured the body fluid volume of the crayfish, *Orconectes virilis,* obtaining values of 25.6 per cent and 25.1 per cent, respectively, with sodium ferrocyanide and a dye, T-1824. The isopods in the present study seemed to have large amount of exoskeleton relative to soft tissue. This fact was further borne out by the relatively low total water values, and in the writer's opinion, supports the estimate of 20 to 30 per cent of total body weight as the body fluid component.

To summarize, it is probable that the osmoregulatory abilities of the experimental animals include a mechanism for active salt uptake and retention. In the experiments conducted at 16° C., the body fluid concentrations and dilutions were not accompanied by detectable weight losses or gains, suggesting that the concentration and dilution are due to salt movement. Since concentrations and dilutions of the body fluids could not be explained purely on the basis of water movement (weight losses or gains), in experiments conducted at 5° C., there is evidence that concentration changes, especially in the higher salinities (75 to 125 per cent sea water) were also due to salt movement at the low temperature. There is some evidence that the experimental animals actively maintain the normal water content of the body fluid. Though body fluid concentrations were well-marked at 16° C., no weight changes were detected. Rather than propose that no water enters or leaves the bodies of the experimental animals upon exposure to the experimental salinities, it might be more reasonable to assume that the normal body water component is actively maintained by pumping water out as fast as it comes in in hypotonic media and by active water uptake and/or salt elimination in hypertonic media. The fact that weight changes were well-marked in experiments conducted at 5° C. and non-existent in experiments conducted at 16° C. indicates that the mechanism for active maintenance of the water balance of the body is depressed or inactivated by low temperature.

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SUMMARY

1. Osmoregulatory requirements were analyzed and compared in Menzies' two subspecies of *Gnorimosphaeroma oregonensis* (*G. o. oregonensis* and *G. o. lutea*) and *Sphaeroma pentodon* Richardson.

2. The mechanism of osmoregulation was studied by measuring changes in the total osmotic concentration of the body fluid after three to 48 hours' exposure to various experimental salinities ranging from fresh water to 125 per cent sea water. Changes were also measured in the field during a partial tidal cycle. The principal findings and conclusions are as follows:

a.) The body fluids of the experimental animals became either diluted or concentrated in the experimental salinities. Generally, in more dilute media (50% sea water or less), the body fluids were maintained hypertonic to the medium, while in more concentrated media (75 to 125% sea water), they were usually maintained hypotonic to the medium.

b.) The lack of weight changes in experimental salinities in experiments conducted at 16° C. indicates that dilution and concentration of the body fluid at normal temperatures are caused primarily by salt movement.

c.) Pronounced weight changes that occurred in experiments conducted at 5° C. suggest that the normal water component of the body fluid is actively maintained and that low temperature interferes with this active maintenance, which normally permits excess water to leave the body in diluted media and to enter in more concentrated salinities. However, the fact that the degree of concentration and dilution of the body fluids at the low temperature could not be explained solely on the basis of water movement suggests concurrent salt gains or losses.

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MOTILITY AND POWER DISSIPATION IN FLAGELLATED CELLS, ESPECIALLY CHLAMYDOMONAS [1]

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The energetics of cellular motion have evoked much interest over the past few decades. Muscle, amoeboid cells, and ciliated or flagellated cells have all been studied, but skeletal muscle has received the most attention. This is true partly because the motion of muscle cells can be stopped and started at the will of the experimenter. This fortunate property, absent in amoeboid and ciliated cells, allows the muscle cell to be compared with itself during rest and exercise. Metabolic poisons can be used to stop movement in non-muscular cells, but chemical inhibition is seldom reversible or specific enough for experimental designs as elegant as those possible in studies on muscle.

Recently, genetic mechanisms have been discovered for controlling the motility of certain flagellated cells: the bacterium *Salmonella typhimurium* (Stocker, Zinder and Lederberg, 1953) and the autotrophic green alga, *Chlamydomonas* (Lewin, 1952). Of the two organisms, *Chlamydomonas* has some advantages as an experimental object, since it is nonpathogenic and has simple, well-defined nutrient requirements. By using ultraviolet light, Lewin (1954) has produced several single-locus mutant strains with abnormal flagellar characters, including some which look just like the wild-type strain but do not move their flagella. The paralysis must be related to an abnormality either of flagellar structure or of some other part of the cell. The failure of Mintz and Lewin (1954) to find serological differences between the flagella of normal and paralyzed strains suggests that these flagella may be structurally similar. If this is so, the loss of motility is probably related to a metabolic change elsewhere in the cell. It is now possible by using these algal strains to compare the metabolism of "normal" and "paralyzed" flagellated cells which are presumably alike in other respects. For this comparison it is necessary to assume that a large and definite proportion of the cells in the "normal" culture is motile. An estimate of this proportion, the motility index, will be developed primarily for use in later studies. Its use in this paper will be only to justify the above assumption.

The energetic cost of flagellar motion will be estimated in two ways. One estimate is based on microscopic study of the motile cells, the other on measurements of respiration. The two estimates will be compared.

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METHODS

The marine organisms used in this study, *Amphidinium Klebsi, Carteria* (?) sp. and *Dunaliella* sp., were obtained from Dr. J. H. Ryther at the Woods Hole Oceanographic Institute. The three strains of *Chlamydomonas* came from the Department of Botany, Indiana University (I.U.): they were *C. Moewusii* (+) (I.U. No. 97) herein called "CMW," *C. Moewusii* (+) (Lewin's paralyzed strain No. M 1001; I.U. No. 697) herein called "CMP," and *C. Reinhardi* (+) (I.U. No. 89, Sager and Granick, 1953) herein called "CRW." Marine organisms were studied in filtered, autoclaved Woods Hole sea water and kept on agar slants made with sea water. Fresh-water *Chlamydomonas* was grown and studied in a liquid medium suggested by Fuller [2] which contained

KNO_3, 1 *M*	5.0 ml.
K_2HPO_4, 1 *M*	0.5 ml.
KH_2PO_4, 1 *M*	0.5 ml.
$MgSO_4$, 1 *M*	2.0 ml.
$Ca(NO_3)_2$, 1 *M*	0.25 ml.
"Trace element solution"	1.0 ml.
"Iron solution"	1.0 ml.
Iron-free water to make	1000 ml.

"Trace element solution" contained

H_3BO_3	1.43 g.
$MnSO_4 \cdot H_2O$	1.05 g.
$ZnCl_2$	0.05 g.
$CuSO_4 \cdot 5H_2O$	0.04 g.
$H_2MoO_4 \cdot H_2O$	0.01 g.
Distilled water to make	1000 ml.

"Iron solution" contained

Disodium ethylenediaminetetra-acetate	0.5 g.
$FeSO_4 \cdot 7H_2O$	5.0 g.
Distilled water to make	1000 ml.

The culture vessels were 125-ml. Erlenmeyer flasks containing 50 ml. of medium and 2.5-liter, wide, flat-bottomed culture flasks (like A. H. Thomas No. 4372-F) containing one liter of medium. Air containing 5% CO_2 was bubbled through the larger cultures. The flasks were shaken mechanically to swirl the contents gently. Four fluorescent lamps (type F40T12W or SW) were mounted under a glass-bottomed water thermostat kept at 23° C., in which the larger flasks were immersed to the level of the medium inside. The illumination 2.5 cm. above the bottom of the flasks was about 500 foot-candles as estimated with a photographic exposure meter. The large flasks were inoculated either with 100 ml. of a previous one-liter culture, or with a 50-ml. culture, reared for the purpose in a small flask. The small flasks were illuminated from above and shaken gently but were not otherwise aerated. One-liter cultures were ready for harvest (about 2×10^6 cells $ml.^{-1}$) in two to four days, depending on the inoculum. The harvest was usually concentrated by gentle centrifugation, and the cells were re-suspended in fresh medium before use.

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[2] R. C. Fuller, personal communication (1955).

Motility of whole populations of cells was studied by comparing photomicrographs of samples of cell suspensions. The film (Du Pont Microcopy) was exposed for 8 seconds and developed for maximal contrast with elon-hydroquinone contrast developer (Kodak formula No. D-11). After being processed, the photographs were projected onto a screen for counting those cells which were stationary long enough to form images. Images of moving cells failed to register because of the long photographic exposure. The use of a haemacytometer[3] and a phase-contrast microscope in photography made the counting easier.

This method leads easily to the formulation of a *motility index*, M. A practical definition of M is

$$M = 1 - \frac{n_1 - n_2}{n_3},$$

in which n_1 = the number of cells counted in a defined area of the photograph of a cell suspension, made with a time exposure of 8 seconds;
n_2 = the number of cells in the first photograph whose images fail to appear in the second, otherwise similar, photograph taken one minute later;
n_3 = the number of cells appearing in a photograph of a different drop of the same suspension, in which all the cells are immobilized (*e.g.*, with HCHO or I_2 vapor).

The second measure of motility used here is based on the speed of locomotion of individual motile cells in a drop of a dilute suspension, placed on a slide and covered with a coverglass, at room temperature (21 to 23° C.). The individuals to be studied were selected at random by tracking every cell which crossed a line bisecting the field, for as long as it remained in the field. The image of the cell was projected on to a sheet of paper, using a camera lucida. The path of motion was described by pencil marks indicating the position of the cell every two seconds. A loudly ticking clock or mechanical sounder was found to be essential. The distance travelled by the cell per second was calculated from a summation of the line segments connecting the pencil marks on the sheet, and from the time elapsed between the placement of the first and the last marks. The distances travelled per second by several cells in the same suspension were averaged to estimate the *average speed of locomotion* for the population.

Oxygen consumption was measured at 23° C. by the Warburg method. Each 14-ml. reaction vessel was inclosed in a light-tight cloth bag and contained 2 ml. of a suspension of cells which had been washed by gentle centrifugation (700 × G, 30 seconds) and re-suspended in fresh medium. The manometers were read every 10 minutes. The respiratory rate was found to decline slowly with time, but not appreciably during the first 90 minutes; the readings during this period were fitted with a straight line by the method of least squares. Respiratory rates were then expressed in μl. of O_2 (S.T.P.) per mg. total nitrogen per hour (Q_{O_2} (N)). Total N was estimated by sulfuric-acid digestion of an aliquot, with three successive additions of H_2O_2, followed by direct Nesslerization and reading of the samples in a Klett-Summerson photoelectric colorimeter (Miller and Miller, 1948).

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[3] A haemacytometer chamber for phase-contrast microscopy is manufactured by the American Optical Co.

RESULTS

Degrees of motility in a culture of C. Reinhardi

When samples of a culture of *C. Reinhardi* were observed with the microscope, they were found to contain some stationary cells. Some of these became motile from time to time; at the same time swimming individuals settled down to become members of the stationary group. In general the stationary group seemed to remain constant in size; therefore, in any series of observations the number of *originally* stationary cells becoming active in any time interval may be expected to bear a constant relation to the number of *originally* stationary cells remaining. To test this supposition, a single drop of a culture was photographed repeatedly over a period of several minutes. The photographs were studied, and numbers of originally stationary cells remaining were plotted on a logarithmic scale against time. In one experiment (Fig. 1) the points fell on a straight line for the first

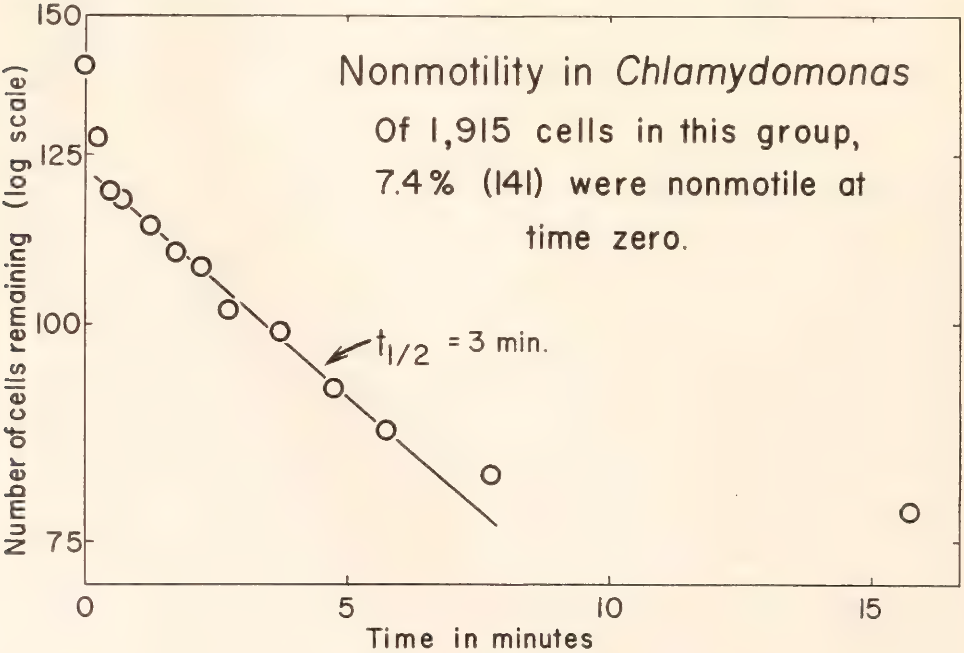

FIGURE 1. Degrees of motility in a culture of *Chlamydomonas Reinhardi*. For the first 5 minutes many of the originally stationary cells became motile as shown by the points fitted with a straight line. During the first 30 seconds a more active group of cells dominated; a slower, possibly more heterogeneous group dominated after 5 minutes.

five minutes; this supports the hypothesis that a constant proportion of the remaining non-motile cells become active during each time interval. However, the graph also revealed that the entire original population of stationary cells was made up of three classes, according to their rates of decrease. The first two had half-times of one and three minutes, respectively. Cells in the third class, possibly including dead individuals in the culture, failed to move in 16 minutes. For this population the first photograph showed 141 (n_1) stationary cells; in the second photo 23 (n_2) of these particular cells were missing. A photo of a killed sample showed 1915 (n_3) cells. Thus, $M = 0.94$. In general, samples from other cultures gave similar results, except that the "one-minute" class often could not be found.

Locomotion of individual cells of several species

A different quantitative concept of cellular motion results from the detailed observation of single motile cells selected at random from a culture. The path of motion of a flagellated algal cell is a series of straight lines or arcs of large radius. Cells may occasionally change direction abruptly or spin briefly in place as if held by a mucous attachment. In addition, cells which are swimming forward often revolve about an axis parallel to the direction of motion (Brown, 1945) and may oscillate as they swim.

For studies of the velocity of motion, several kinds of elliptical or nearly spherical flagellates were selected. Table I shows the observations and calculations derived from them. The "average radius" is one fourth the sum of length and width. The minimal power dissipation, P, per cell was calculated from Stokes's Law relating to the force, f, needed to propel a sphere through a fluid:

$$f = 6\pi r \eta u,$$

and from the relation

$$P = 10^{17} fu,$$

where

f = force needed to overcome fluid resistance (dyne),
r = average radius of cell (cm.),
η = viscosity of fluid (poise = dyne sec. cm.$^{-2}$),
u = average speed of locomotion (cm. sec.$^{-1}$),
P = power (watt = 10^7 dyne cm. sec.$^{-1}$).

Oxygen consumed by normal and by paralyzed Chlamydomonas

A third aspect of flagellar motion concerns the intensity of metabolism of the flagellated cell. In this study, the oxygen consumption of a population of normal *Chlamydomonas Moewusii* (CMW) was compared with that of the ultraviolet-induced, "paralyzed" mutant (CMP). The mutant cells have flagella but fail to use them; these are held out rigidly almost perpendicular to the main axis of the cell. Occasionally a flagellum showed a little motion at its tip, but this hardly ever caused the whole cell to move.

The figures in Table II are based on 12 reaction vessels for CMW and 13 for CMP. In preparation for each experiment the cells of the two strains were reared

TABLE I

Minimal power output of selected flagellated cells

Species	Size		Medium	Aver. velocity 10^{-2} cm. sec.$^{-1}$	Min. power output/cell, 10^{-16} watt
	Aver. radius 10^{-4} cm.	Length / Width			
Amphidinium Klebsi	7.98 (11)	1.30	Sea water	0.739 (11)	7.5
Carteria (?) sp.	6.54 (9)	1.67	Sea water	1.25 (10)	17
Chlamydomonas Moewusii (CMW)	5.54 (11)	1.48	Fresh water	1.28 (16)	15
Dunaliella sp.	4.40 (9)	1.35	Sea water	2.26 (9)	38
Chlam. Reinhardi (CRW)	3.26 (100)	1.08	Fresh water	0.828 (100)	3.9

Numbers of individuals studied are in parentheses. Viscosities (corrected for density) used in calculations were: sea water, 0.965 cp (estimated from Miyake and Koizumi, 1948); fresh water, 0.931 cp.

in one-liter cultures under identical conditions. In each experiment the oxygen consumption of the paralyzed cells was less than that of the normal cells when expressed in terms of total cellular nitrogen.

DISCUSSION

The quantitative description of cellular motility will be discussed before considering the energy required for flagellar motion. This study presents two quantitative methods of studying locomotion in populations of flagellated cells.

The *motility index* (photographic method) can be used for distinguishing the behavior of cell populations exposed to varying experimental treatments. It may prove helpful in pharmacologic and toxicologic studies on suspensions of algae, protozoa, bacteria, or sperm cells; these forms may offer the experimenter advantages over larger and more expensive animal subjects. Compared with other proposed estimates of the proportion of non-motile cells in a microscopic field (Emmens, 1947; White, 1954) it would appear to avoid certain subjective errors in sampling and counting, and to minimize the error due to the inclusion of cells

TABLE II

Oxygen consumption (Q_{O_2} (N)) of normal and paralyzed Chlamydomonas Moewusii (paired comparison)

Experiment no.	CMW	CMP	Difference (CMW-CMP)
6-27-57	1.24	1.14	0.10
7- 1-57	1.36	1.24	0.12
7-22-57	1.31	0.79	0.52
8- 3-57	1.19	1.14	0.05
8- 5-57	2.33	2.06	0.27
8- 7-57	2.00	1.72	0.28
Mean difference and its standard error ($n = 6$)			0.223 ± 0.0706

which may stop for momentary "rest" periods. It cannot distinguish degrees of impairment of locomotion.

The *average speed of locomotion* appears to be valuable for distinguishing populations of cells which show normal speeds of locomotion from populations with impaired locomotion. It takes no notice of non-motile cells, and thus becomes most useful in estimating the degree of motility in cultures where the motility index is high. It is similar in principle to one devised by Baker, Cragle, Salisbury and Van Demark (1957) who measured the time required for 100 free-swimming sperm cells to pass through a segment of a plane. Their method, which seems admirably suited to cells displaying the sperm type of locomotion, has the advantage of presenting the result of an experiment immediately without waiting for photographic processing. The decision to use a given method will rest partly upon the extent to which its assumptions are fulfilled by the swimming habits of the organism. The method described here is of special value, since from it can be derived an estimate of the external work done by the motile cells in the population.

The estimates of power dissipation in Table I are certainly low, because the premises on which they are based all tend to reduce the estimates. It is supposed, for example, that the cell's internal energetic conversions are 100% efficient. The other assumptions, each known to be false to some extent, are: that there are no degrees of motion other than uniform in a straight line (contradicted by Brown, 1945, and others), that the cell is a sphere (contradicted in Table III), and that the frictional drag of the flagellum, apparently of major importance in the locomotion of sea urchin sperm (Gray and Hancock, 1955), is negligible in *Chlamydomonas*. Excepting *C. Reinhardi*, the smaller flagellates travelled faster and displayed a higher power output than the larger cells. Whether this difference is related to a greater metabolic rate of the smaller cells has not been determined.

TABLE III

Estimates of size of Chlamydomonas

Strain..........	CRW	CMW	CMP
Length, μ	6.49 ± 0.14	7.64 ± 0.17	7.41 ± 0.14
Width, μ	5.24 ± 0.17	5.62 ± 0.14	4.92 ± 0.10

The "±" sign is inserted between the mean and its standard error. Fifty cells of a single culture of each strain were measured.

When normal and paralyzed *C. Moewusii* were reared and studied under the same conditions in several successive experiments, the paralyzed cells (CMP) always consumed less oxygen than did the wild-type, motile cells (CMW). The average difference in Q_{O_2} (N) was about 14% of that of the normal cells, and was found to be statistically significant (*t*-test, $n = 6$) at the 5% level, but not at the 2% level. It must be assumed that the proportion of dead cells in the CMP culture is no greater than in the CMW culture. In interpreting this difference, certain other features of the two strains should be borne in mind.

Ocular micrometer measurements showed that although CMW and CMP are of equal length, the paralyzed cells are, on the average, a little more slender than the motile ones (Table III). Thus, a paralyzed cell's surface-to-volume ratio is slightly greater than that of a normal cell. From the size of this difference alone one would expect the Q_{O_2} (N) of the paralyzed strain to be a little greater than that of the normal strain; it appears in fact to be less. The single mutation which resulted in paralysis of the flagella may have had other expressions, possibly involving alterations in the efficiency of biochemical pathways of metabolism. In summary, the physiologic differences between the two strains may be much greater than appeared at first. In ignorance of the magnitudes of these possible factors, it is tempting to suggest that the difference in oxygen consumption is actually related to the state of motility of the cell, but a cautious attitude seems desirable.

As a partial test of this relationship, we may now compare the two available estimates of the energy required for motility. One of these (Table I) states that *C. Moewusii* dissipates at least 10^{-15} watt per cell in overcoming the frictional losses in water. The other estimate is derived from the difference in Q_{O_2} (N) between the normal and paralyzed strains, which is 0.22 μl. hr.$^{-1}$ (mg. N)$^{-1}$. If we suppose that the exclusion of light from the Warburg vessel does not affect motility (Lewin, 1953), the two figures are comparable; the latter figure can then be transformed to watts per cell by making the following reasonable assumptions:

1. The consumption of 1 μl. of O_2 releases about 4.8×10^{-3} calorie or 5.58×10^{-6} watt hour.
2. A CMW cell contains 2.65×10^{-9} mg. N (estimated from cell counts and N determinations on a single culture at the time of harvest).
3. The motility index in the CMW culture is high.

The observed difference in the rate of oxygen consumption thus corresponds to a difference in power dissipation of 3.3×10^{-15} watt per cell. Rothschild's (1953) reworking of Taylor's figures gives estimates close to these for the minimal energy dissipated by bull sperm: for two kinds of assumptions, 3.74×10^{-14} watt and 2.04×10^{-15} watt per cell. In our comparison, the efficiency of conversion of chemical to mechanical energy is not taken into account. The closeness of the two estimates derived in this paper suggests that the lower rate of oxygen consumption of paralyzed cells may be correlated with their loss of motility.

SUMMARY

1. The paper describes a method for estimating the minimal power output of individual, nearly spherical, flagellated cells. A comparison of 5 species of green flagellates suggests no relationship between size and power dissipation (Table I).

2. A simple photographic method for estimating the fraction of motile organisms in a culture is described.

3. Cultured populations of *Chlamydomonas Reinhardi* may contain two or more distinct groups of cells with different degrees of motility (Fig. 1).

4. The motile, "wild-type" *C. Moewusii* consumed 1.57 μl. O_2 (S.T.P.) per hour per mg. total N.

5. A paralyzed mutant strain of the same species consumed 14% less oxygen than the wild type. The extra oxygen consumed by the motile strain is commensurate with its estimated minimal power output.

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CONSEQUENCES OF UNILATERAL ULTRAVIOLET RADIATION OF SEA URCHIN EGGS[1]

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The suppression of the elevation of the fertilization membrane on the half of a sea urchin egg which directly receives high doses of ultraviolet light has been described by Reed (1943) and Spikes (1944). The experiments reported herein are an examination of the consequences of unilateral U.V. irradiation of the sea urchin egg in terms of changes in cell morphology with dose, the physical state of the cytoplasm, the effects of time and temperature, and the effects on subsequent cell division. Particular attention is directed toward observations on hyaline layer formation, local gelation, and excentric formation of the mitotic figure.

MATERIALS AND METHODS

Gametes were obtained from the sea urchin *Strongylocentrotus purpuratus* by injection with 0.5 *M* KCl. The groups of eggs selected were more than 99% fertilizable, were free from visible abnormalities, yielded symmetrical fertilization membranes, and showed little distortion when the lifting of the fertilization membrane began. The pattern of morphological changes at different doses was confirmed with suitable eggs obtained from a single female of the related species *Strongylocentrotus franciscanus,* which has larger eggs with less yolk.

The ultraviolet source was an Electrotherapy Products Corp. low pressure mercury vapor lamp, which produces approximately 95% of its U.V. energy in a 2537 Å band. The intensity was measured with a Hanoviameter.

In some experiments the eggs were centrifuged in a Servall refrigerated anglehead centrifuge, either in sea water or in a sucrose gradient formed by layering sea water over 0.88 *M* sucrose.

Unless otherwise noted, all experiments were carried out in 1 cm. deep, filtered sea water at 17.5 ± 0.1° C. Artificial calcium-free sea water was prepared according to the formula of Moore (1956).

Clarification of terminology

In order to describe concisely and accurately the changes associated with unilateral irradiation of the strongly-absorbing egg certain special terms must be defined. The *directly-irradiated hemisphere* is the surface of the egg which faces the U.V. lamp. The *shaded hemisphere* is the surface which does not face the lamp, and, hence, is shaded by the cytoplasm. The *shaded-irradiated axis* is an imaginary line drawn between the *poles* or centers of these two hemispheres. *Unilateral membranes* are fertilization membranes which lift off the egg on the shaded hemisphere only. All drawings and photographs except Figures 1 and 8 have been mounted with the shaded pole facing the top of the page.

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[1] Supported by grants from the American Cancer Society and the Office of Naval Research awarded to Dr. Daniel Mazia.

[2] This work was performed under the tenure of a Research Fellowship of the National Cancer Institute, United States Public Health Service. Present address: Department of Biological Sciences, Florida State University, Tallahassee, Florida.

RESULTS

When eggs were irradiated with large doses of U.V. and then fertilized, the height of the fertilization membrane and the hyaline layer on the directly-irradiated hemisphere was reduced. Sufficiently large doses unilaterally inhibited the formation of these membranes entirely.

The dose required to produce a definable level of effect varied by as much as a factor of three between the most sensitive and the most resistant groups of eggs. Nevertheless, the ratio of doses necessary to produce two definable effects on the majority of eggs in a population appeared to be constant even in the extreme cases. The data presented represent the most frequently encountered dose relations.

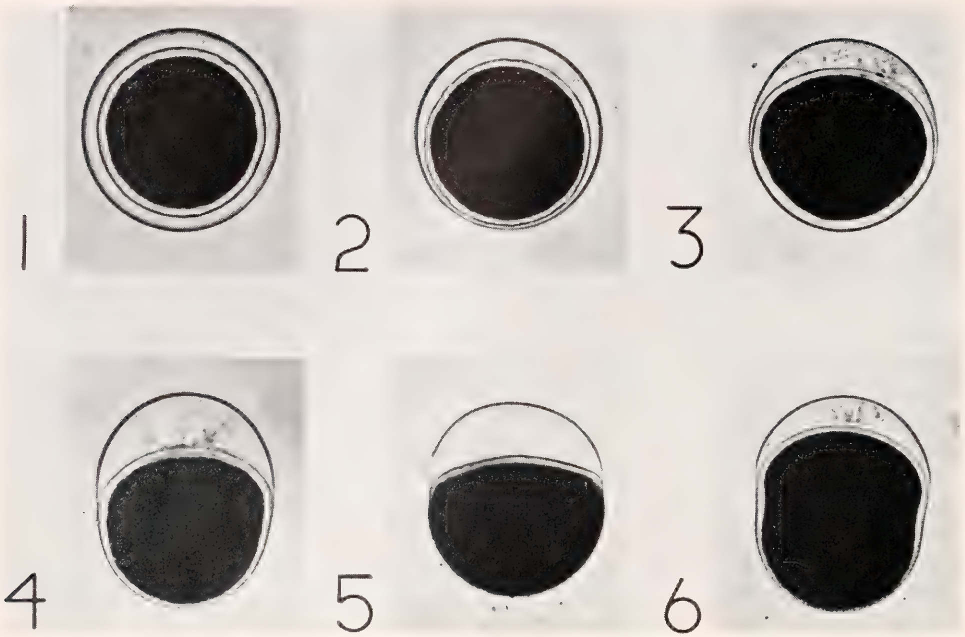
Less than 1600 ergs/mm.^{2} did not interfere with the normal membrane elevation. When the dose was increased the fertilization membranes did not elevate to their normal height over the irradiated pole (Fig. 2). Doses of approximately 2800 ergs/mm.^{2} resulted in the almost complete suppression of the fertilization membrane over a small area, but the hyaline layer differentiated over the entire surface. When the dose was increased to 4800 ergs/mm.^{2} the fertilization membrane covered only one hemisphere, while the hyaline layer appeared normal (Fig. 3). With slightly higher doses a reduction in the thickness of the hyaline layer was sometimes found (Fig. 4). With doses above 7200 ergs/mm.^{2} the hyaline layer could be distinguished only slightly beyond the cell equator (Fig. 5). No further changes in the pattern of membrane elevation were noted at increased doses up to the range of 40,000 to 50,000 ergs/mm.^{2} At this dose level partial cytolysis often occurred immediately on the directly-irradiated hemisphere, and complete cytolysis usually followed after standing or at fertilization.

Identification of the inhibited surface

A simple experimental procedure was devised to demonstrate that the irradiated surface was in fact the one that showed inhibition at fertilization. Stationary eggs were irradiated from above with 7200 ergs/mm.^{2} in a large petri dish on a microscope stage and observed as sperm were carefully added. In four experiments there was no detectable net rotation of any of the eggs in the field of a low power objective. By careful focussing it was established that the fertilization membranes first encircled the lower hemispheres which had been shaded by cytoplasm. As the membranes raised further the eggs rolled over and came to rest on their sides revealing total suppression of membrane elevation on the irradiated hemispheres.

Relationship to time and temperature

Eggs were fertilized at regular intervals from a few seconds after irradiation to as much as twelve hours later without any visible changes in the unilateral



FIGURES 1 to 6

Photomicrographs of sea urchin eggs showing different degrees of suppression of the fertilization reaction when irradiated with increasing doses of U.V. from the direction of the bottom of the page.

FIGURE 1. Control.

FIGURE 2. Reduction of the height of the fertilization membrane.

FIGURE 3. Complete suppression of the elevation of the fertilization membrane on the directly-irradiated hemisphere.

FIGURE 4. Reduction of the height of the hyaline layer.

FIGURE 5. Complete suppression of both fertilization membrane elevation and the hyaline layer differentiation on one hemisphere.

FIGURE 6. Later swelling of the initially flattened shaded hemisphere of an egg similar to Figure 4.

fertilization reaction. In five separate experiments there was no increase or decrease in the inhibited area with time. In general, the irradiated eggs cytolyzed sooner than the controls, but in most experiments both the irradiated and the control eggs became unfertilizable at approximately the same time, even with very high concentrations of sperm.

Attempts were made to re-fertilize eggs which had been fertilized but did not completely differentiate the hyaline layer. The simple addition of viable sperm did not cause re-fertilization at any time up to 28 hours after irradiation. The sperm were observed to accumulate in the egg jelly which adhered to the irradiated hemisphere in each of these experiments.

Irradiating eggs from the same females at 18 and 8° C. with various doses revealed that there were no differences in sensitivity at the two temperatures.

Changes in morphology and physical state of the cytoplasm

The progressive dose-dependent suppression on the elevation of the fertilization membrane and the differentiation of the hyaline layer have already been described. Sometimes at high doses the fertilization membrane was elevated to an abnormal height above the shaded pole and the cytoplasm under it was considerably flattened (Figs. 4 and 5). A large amount of particulate matter, possibly cortical granule materials, was found in the perivitelline space under these conditions. The amount of this material was apparently greater at all doses than in the controls.

After flattening, the cytoplasm under the unilateral membranes sometimes swelled and reduced the thickness of the perivitelline space (Fig. 6). In some cases the thickness was less than the controls. Under these conditions there was a constriction around the cell at the equator where the fertilization membrane met the hyaline layer (Fig. 6).

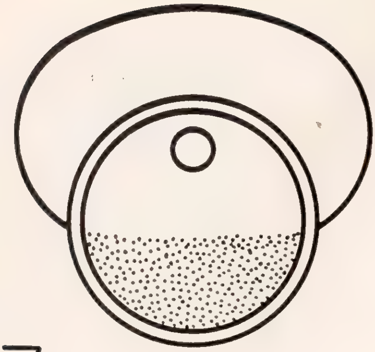
There were no cases of membrane elevation activation by U.V. at any dose in any of the experiments.

Unfertilized irradiated eggs were centrifuged for ten minutes at approximately 12,000 g in a sucrose gradient. In two such experiments 90% of the eggs stratified with the center of the light pole (identified by an oil cap over a clear region of cytoplasm) in the center of the shaded hemisphere (identified by subsequent fertilization) (Fig. 7). Almost all of the remaining 10% had an asymmetry of less than 30° between the light-heavy and the shaded-irradiated axis. A very small fraction of a per cent were 30 to 90° off center, and no cases were found in which the shaded pole appeared to have a greater density than the irradiated one.

When unfertilized eggs were placed in 70% sea water after irradiation they swelled on one pole only, giving the eggs a somewhat pear-shaped appearance. Standing in this hypotonic medium for several hours did not result in any further changes in shape. The treated eggs were fertilized to establish that the shaded pole was the swollen one. Hence, while both unirradiated eggs and the shaded side of an irradiated one swell in 70% sea water, the directly irradiated surface does not.

Irradiated eggs placed in 70% sea water had a dense darkened area near the irradiated pole, a somewhat less dense region at the shaded pole, and a lighter less granular region near the equator. Occasionally this pattern appeared in eggs kept in normal sea water and seemed to be accompanied by a slight enlargement of the shaded hemisphere. With doses of the order of 40,000 ergs/mm.^{2} a large blister of non-granular material formed on the irradiated pole when the eggs were placed in the hypotonic sea water. With slightly higher doses these blisters appeared spontaneously.

Irradiated eggs were centrifuged at approximately 12,000 g in sufficiently dense suspensions that some of the eggs were confined in a random orientation with respect to their light-heavy axes. Some of these cells showed stratification only on the shaded side, which was identified by subsequent fertilization. When the direction of centrifugation was perpendicular to the shaded-irradiated axis there was a narrow region near the irradiated surface with a very high gel strength that resisted stratification when the central cytoplasm and the shaded side stratified (Fig. 8).



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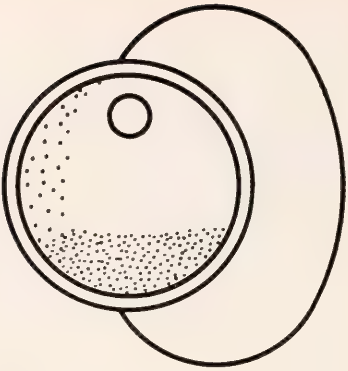

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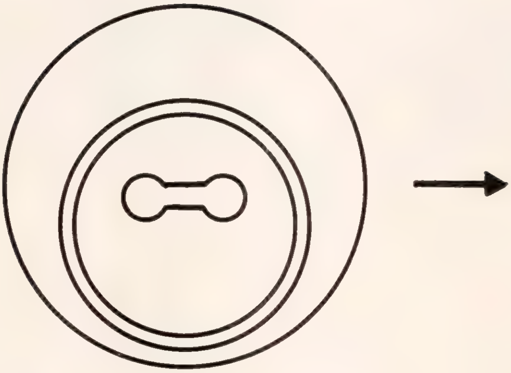

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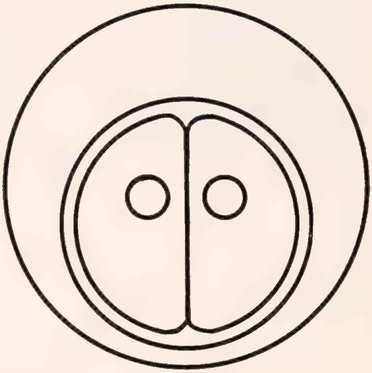

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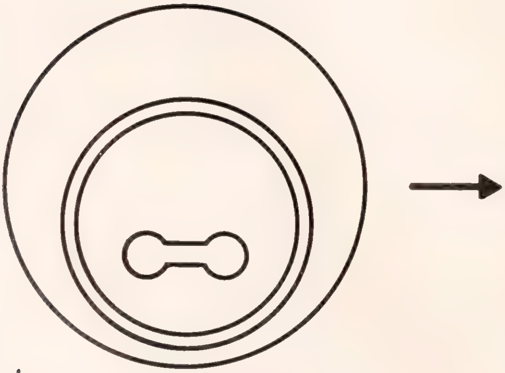

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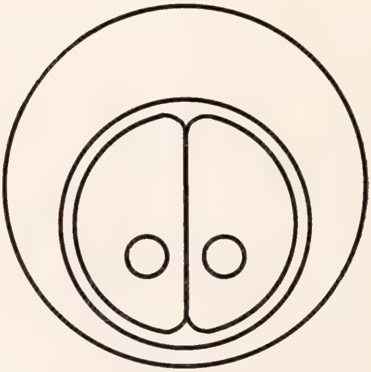

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Figures 7–12

Eggs irradiated after equilibration in calcium-free artificial sea water and fertilized immediately when returned to normal sea water showed the same degree of inhibition as eggs irradiated in normal sea water.

Mitotic abnormalities

Cells irradiated at doses that inhibited the full differentiation of the hyaline layer seldom divided. At lower doses some or all of the eggs would divide several times and sometimes form apparently normal swimming blastulae. Gastrulation was usually abnormal. In some experiments even the first division was abnormal.

A systematic group of abnormalities occurred as a result of the mitotic figure failing to migrate to the center of the egg. The nucleus of the unfertilized egg is excentrically located, and in normal division the mitotic apparatus is *positioned* approximately in the center of the cell. The position of the furrow is determined by the plane formerly occupied by the metaphase plate both in normal cells and these abnormal cells.

When the mitotic figure located in either hemisphere was oriented perpendicular to the shaded-irradiated axis, the furrow formed along that axis and the egg cleaved into two equal-sized blastomeres (Figs. 9 to 12).

When the mitotic figure was oriented parallel to the shaded-irradiated axis in either hemisphere, the furrow formed perpendicular to the axis and the sizes of the resulting blastomeres were quite different (Figs. 13 to 16).

Variable results were observed when the mitotic figure was formed with other orientations with respect to the shaded-irradiated axis (Figs. 17 and 18).

Excentric spindles were also found in eggs which were irradiated during the early part of the first mitotic cycle with comparatively low doses of U.V. The blastomeres in such experiments were always equal in size.

Whenever the mitotic apparatus was excentric the furrow formed first on the surface that was closest to the spindle. At later stages of cytokinesis the furrow on the near side would always be deeper than the furrow on the far side. In some cases the furrow actually passed through the spindle before the first indentation occurred on the far side of the cell.

DISCUSSION

The progressive unilateral inhibition of the fertilization reaction has been described in terms of the U.V. doses required to produce different degrees of inhibi-

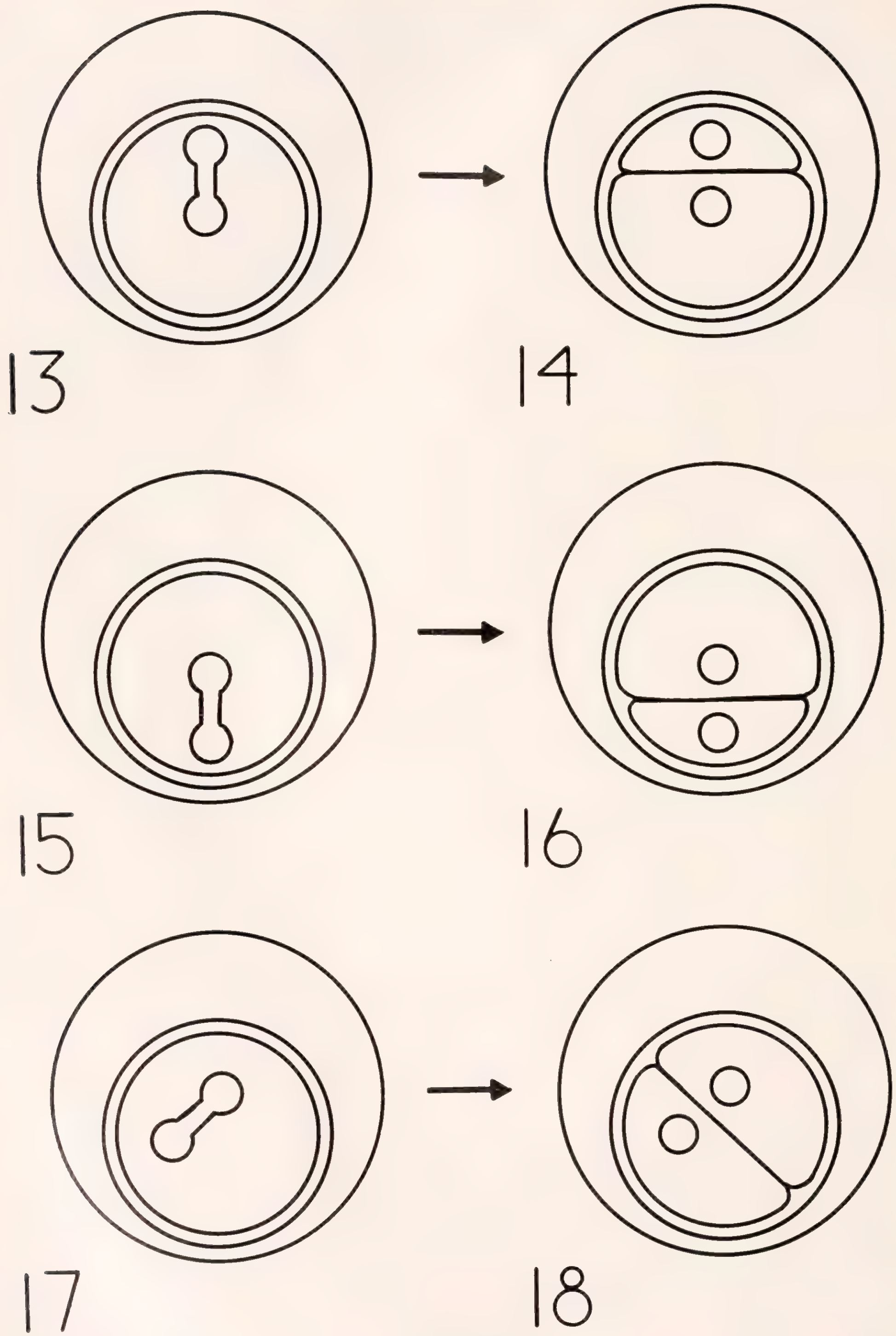
FIGURES 7 to 18

Schematic drawings of eggs irradiated from the direction of the bottom of the page (except Fig. 8); refer to text for explanation.

FIGURE 7. Egg centrifuged in a sucrose gradient and then fertilized. Stratification direction indicates that the irradiated pole was heavier than the shaded pole.

FIGURE 8. Egg irradiated from the left side of the page and centrifuged while confined with the shaded-irradiated axis perpendicular to the direction of centrifugation. A narrow region near the surface of the irradiated hemisphere resisted stratification indicating a local increase in gel strength.

FIGURES 9 to 12. Division patterns of cells with spindles oriented perpendicular to the shaded-irradiated axis.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Figures 13–18

tion of both the elevation of the fertilization membrane and the differentiation of the hyaline layer. Hyaline layer differentiation is less sensitive to U.V. than fertilization membrane elevation; however, it may be suppressed completely on the directly-irradiated hemisphere with high doses. The inhibition of the elevation of the fertilization membrane has been described previously by Reed (1943) and Spikes (1944).

By means of local dye experiments Spikes (1944) was able to demonstrate that the directly-irradiated hemisphere is the site of inhibition. His findings have been reconfirmed with the direct observations of undisturbed eggs reported herein. Giese (1947) has shown that the sea urchin egg strongly absorbs or scatters 2537 Å U.V. light. Harvey and Lavin's (1944) U.V. photomicrographs also indicate that a considerable amount of the light is absorbed in sea urchin eggs of another genus. Since the shaded pole is not inhibited even at very high doses, it may be concluded that the transmission of the cytoplasm is too low to allow the necessary energy to reach the sensitive sites on the shaded side of the egg.

The demonstration that there was no spreading of the damaged area with time indicates that the U.V. action is relatively direct, and, in particular, that there is no secondary effect of "diffusible poisons." There was no recovery with time; hence, the damage seems to be irreversible by any metabolic mechanism. Since the degree of injury did not decrease with time, and a diffusible toxic product would be expected to decrease in local concentration, this observation provides additional evidence against the action of such substances.

The sensitivity was the same at 8 and 18° C.

Direct photochemical action has been shown repeatedly to have a Q_{10} of approximately 1. Therefore, insofar as visually equivalent degrees of damage may be used as a measure of the rate of damage, it appears that the injury results from direct photochemical action. The time and temperature relations together offer evidence that the effect is localized and that there is a lack of intermediary toxic products.

The observation that the inhibited surface could not be re-fertilized by the addition of fresh sperm could be interpreted in two ways: either the U.V. damage rendered it unfertilizable or some of the steps of the fertilization reaction occurred on this side when the egg was initially fertilized. If some substances necessary for the initial steps of the reaction had been used up the sperm could not initiate a response later. A pronounced green Becke line appears in the out-of-focus image of the damaged hemisphere of heavily irradiated eggs after fertilization. This change is probably similar to the dark-field changes which have been observed prior to membrane elevation (Runnström, 1928; Rothschild and Swann, 1949) and indicates that some step in the fertilization reaction has taken place.

Two types of evidence for local gelation in the irradiated hemispheres were obtained: first, that swelling in 70% sea water was confined to the shaded pole, and, second, that a narrow band near the irradiated surface resisted stratification with centrifugation when the rest of the cytoplasm stratified. Reed (1948) found

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FIGURES 13 to 16. Division patterns of cells with spindles oriented parallel to the shaded-irradiated axis.

FIGURES 17 and 18. An example of one of several division patterns obtained when the spindles have intermediate angular orientations.

that moderate doses of unilateral U.V. did not change the permeability of the egg to a large variety of solutions. Although no measurements were made, he discussed possible differences at higher doses and proposed that some sort of gelation occurred on the basis that vacuoles were formed in the irradiated pole. Spikes (1944) also proposed that gelation occurred, because he found that while normal eggs only swelled in 50% sea water, irradiated ones lysed on the irradiated side.

Spikes' data might also be interpreted as indicating either that the surface of the shaded hemisphere was weakened or that the osmotically inert volume had been increased permitting greater than normal swelling followed by lysis. The observation of the large amounts of granular material released into the perivitelline space at the shaded pole suggests the weakening either of the cell membrane or of some other surface structure. The flattening of the shaded pole at fertilization at high doses seems to fit either hypothesis, although an enhancement of the vigor of the fertilization reaction would yield the same pattern. It would not be unreasonable to suppose that U.V. damage could affect both the surface strength and the osmotically inert volume, perhaps by a common mechanism.

The observation that eggs irradiated in calcium-free sea water showed the same degree of damage as eggs in normal sea water cannot be interpreted directly in terms of the often demonstrated role of calcium in gelation (Heilbrunn, 1952). First, the eggs had to be fertilized in normal sea water since fertilization will not occur in the absence of external calcium ion; hence, new calcium may have been introduced before the damage was measured. Second, since Heilbrunn and his co-workers have shown that U.V. causes solation in low doses and gelation in high doses, it is quite possible that the calcium ion left in the egg after treatment with calcium-free sea water shifts between the less and more heavily damaged portions of the cytoplasm. The second possibility is quite attractive, since it would provide a mechanism for an increase in osmotically inert volume in the less damaged hemisphere and introduces the possibility that the surface on the shaded side might be weakened by small amounts of U.V. penetrating the cytoplasm to cause solation.

Spikes (1944) reported that in *Lytechinus pictus* furrow formation almost always occurs along the shaded-irradiated axis. Clearly this is not the case in the *Strongylocentrotus purpuratus* used in these experiments; cleavage may take place with any orientation. Successful cleavage with the furrow passing through the irradiated portion of the egg indicates either that the furrowing strength exceeds the resistance of the radiation-induced gel or that the gel is solated in the course of cytokinesis.

Cleavage into equal or unequal sized blastomeres is determined by the orientation of the spindle with respect to the shaded-irradiated axis. It occurs because the mitotic figure remains centered around the original location of the nucleus. The nucleus is excentrically located in unfertilized eggs of this species. When the axis of the mitotic figure is perpendicular to the shaded-irradiated axis the blastomeres are equal in size. Where the axes are parallel the blastomeres are unequally sized. In intermediate angular orientations the results are variable. While both parallel and perpendicular orientations can occur when the mitotic figure is located in either the shaded or irradiated hemisphere, mitotic figures near the equator seem to be restricted to intermediate angular orientations. It is clear that the migration of the nucleus to its normal central position is inhibited. An increase in cytoplasmic viscosity would provide a plausible explanation for this failure of migration.

It is a great pleasure to acknowledge my gratitude to Professor Daniel Mazia for his helpful advice and encouragement during the course of this work. I also wish to thank Professors J. E. Gullberg, L. V. Heilbrunn and C. B. Metz for their valuable comments about the results, and Mr. Fred Burnet for his skillful preparation of the drawings.

SUMMARY

1. The progressive dose-dependent inhibition of the fertilization reaction on the directly-irradiated hemisphere of the unilaterally U.V.-irradiated sea urchin egg has been described in terms of changes in the ability to elevate the fertilization membrane and to differentiate the hyaline layer.

2. Membrane elevation was not activated by 2537 Å U.V. light.

3. No spreading of the extent of injury or recovery was found with time; and no temperature sensitivity differences were found; hence, the injury appeared to be the result of direct photochemical action.

4. The irradiated hemisphere of the fertilized egg maintained its jelly for considerable periods of time.

5. Evidence was obtained showing partial gelation of the irradiated hemisphere and suggesting that the gelled cytoplasm had a higher density than the rest of the egg. Irradiation in calcium-free sea water did not change the degree of damage observed after fertilization in normal sea water.

6. The behavior of the cytoplasm of the shaded hemisphere at fertilization suggested either that the surface structure was damaged or that the osmotically inert volume had been increased.

7. Unilateral irradiation caused excentric spindle formation which resulted in equal sized blastomeres if the spindle axis was perpendicular to the axis of irradiation and unequal sized blastomeres if the axes were parallel.

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THE ROLE OF THE INITIATOR CELL IN SLIME MOLD AGGREGATION [1]

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Previous studies of slime mold aggregation (Sussman and Noel, 1952) had shown that the number of aggregative centers is linearly related to the number of cells present and, further, that centers are distributed in accord with the Poisson series among small, replicate population samples. These and supporting data were considered to dictate the existence of specially endowed individuals termed "initiator cells," each of which could evoke the aggregative response by its neighbors, the "responder cells." Recently a distinctive cell type was detected by morphological criteria in *Dictyostelium discoideum* Raper and evidence was presented in support of the contention that cells of this type are in fact the initiators of aggregation (Ennis and Sussman, 1958a, 1958b; Sussman, 1958). The distinctive individuals, termed I-cells, are much larger than the remainder of the population (R-cells), the difference amounting to 2–3-fold in diameter, 3–10-fold in area. They are much flatter and more heavily granulated and vacuolated. In contrast to the R-cells which move sluggishly, the I-cells are highly motile and extensive lobopodia and filopodia are seen to protrude constantly and explosively. Figure 1 presents histograms to illustrate the size differences. Two modes are apparent without overlap.

The evidence (Ennis and Sussman, 1958b) supporting the candidacy of the I-cells for the appellation of "initiator" is summarized below:

a) The ratio of I-cells to R-cells remained constant during the pre-aggregative period at 1:1940. This figure agrees closely with the ratio of centers formed to cells present at optimal density (1:2200).

b) A high correlation was encountered between the positions of I-cells and of subsequently formed aggregative centers.

c) The appearance of centers among small, replicate population samples was correlated (perfectly in one experimental series and almost perfectly in another) with the previously determined incidence of I-cells. That is, centers appeared in samples containing I-cells; none appeared in samples without I-cells.

d) Removal of I-cells at an early enough time prevented subsequent center formation.

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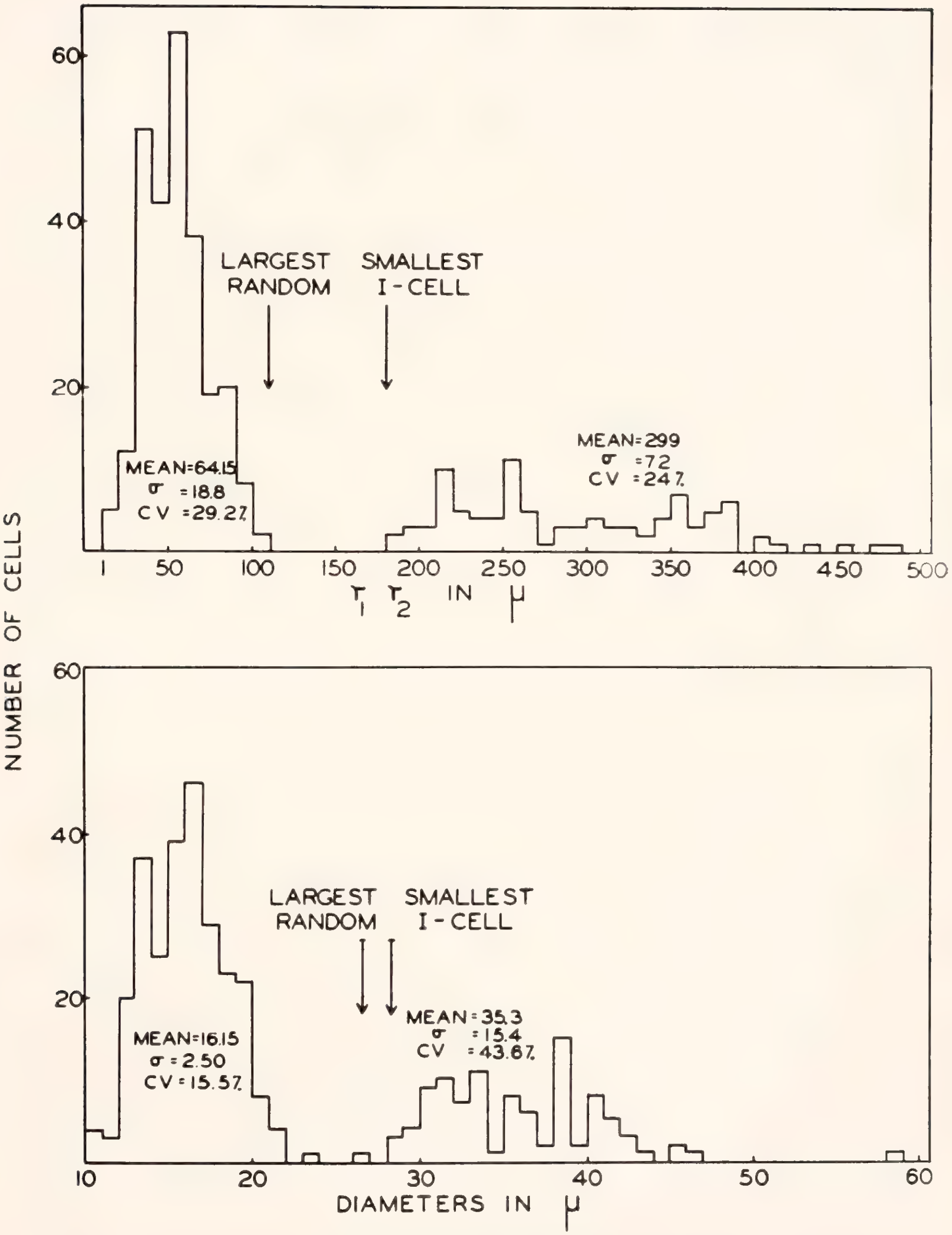

FIGURE 1. Histograms of mean diameters and products of major and minor radii. I-cells were detected under 100 × and confirmed under 440 × as described in the Methods section. As controls, myxamoebae were chosen at random for micrometric determinations.

e) Micromanipulation of I-cells to test areas caused the induction of aggregates within the test populations, whereas movement of R-cells at the same stage of development did not.

The data to be presented provide subsidiary support for the contention that the I-cells are the initiator cells and throw light upon their role in the aggregative sequence.

METHODS

D. discoideum, strain NC-4 wild type, was grown on SM agar medium in association with *Aerobacter aerogenes* (Sussman and Noel, 1952). After 44 hours at 22° C., the myxamoebae had attained the stationary phase and were harvested, washed by differential centrifugation, and dispensed on washed agar plates (Sussman and Noel, 1952). Under these conditions the myxamoebae do not increase in number and aggregate and fruit in normal fashion.

The procedure for I-cell identification has been previously given in detail (Ennis and Sussman, 1958b). Initial recognition is accomplished at 100 × magnification by size and flatness. Examination at 440 × reveals the great pseudopodial activity, high rate of protoplasmic streaming, and granulation, and thereby confirms the diagnosis. A cautionary note is appended here. Occasionally, one encounters moribund cells which typically attain enormous size before lysing. However, these cells are perfectly round and hemispherical. They display no motility and have lost their granules and vacuoles. After one has seen an I-cell, there is no chance of confusing the two types and in any case, moribund cells are extremely rare under the conditions of preparation and incubation described above.

RESULTS

A. Time-lapse studies of aggregation

As mentioned, a high correlation was shown to exist between the positions of I-cells and of subsequently formed aggregative centers. In these experiments, washed myxamoebae were dispensed on washed agar at a population density of 200 cells/mm.^{2}, optimal for center formation in this strain (Sussman and Noel, 1952). After 8 hours' incubation at 22° C., low power fields were chosen at random and fixed in position on microscope stages. Those found to contain I-cells were retained for further study. Camera lucida drawings or photomicrographs were made at intervals until aggregation had begun and the centers were established. In 50% of the fields, a center formed precisely at the position of the I-cell. (Since in this stock, the ratio of centers formed to cells present is 1:2200, the random chance of predicting that a center would form at a particular cell is 0.05%.) In 30% of the fields a center formed near the I-cell. No centers formed in the remaining 20%. In contrast, the incidence of centers in randomly chosen fields, not examined for the presence of I-cells, was 25%. Thus the over-all chance of a center appearing in a field containing an I-cell was three times greater than random. The numerical data, given in detail elsewhere (Ennis and Sussman, 1958b), are here amplified by time-lapse series of camera lucida drawings and photomicrographs.

Figure *2* illustrates the sequence of events when the center formed at the I-cell. The first overt sign of impending aggregation was the appearance of large cell clumps near the I-cell. Associations of more than two or three cells were never encountered prior to this time and even these were purely transient. In the series shown, the I-cell itself became part of a clump as its nearest neighbors began to

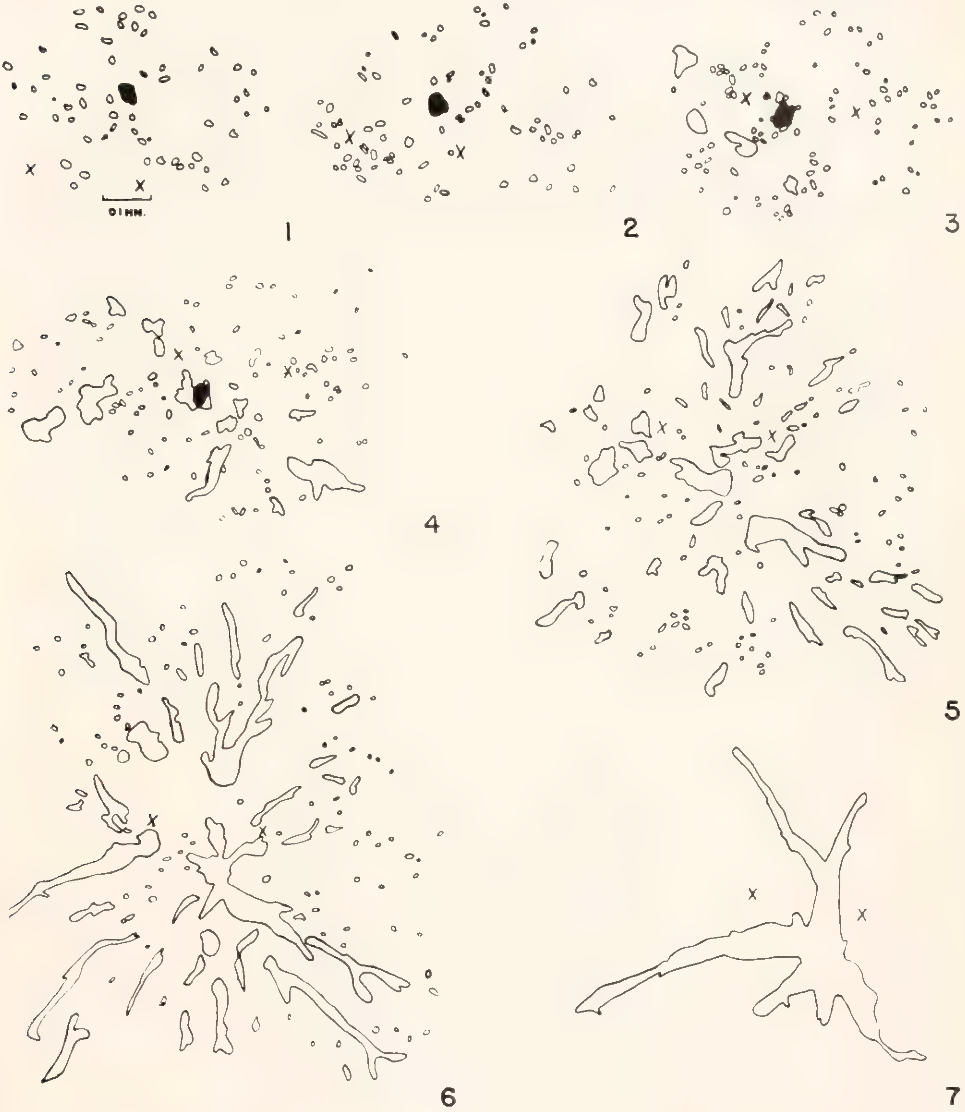

FIGURE 2. Time lapse camera lucida drawings of aggregation. The I-cell is the black individual. Cell clumps, appearing first in C, were merely outlined. The two cross-hatches mark the positions of dirt particles, used as points of reference. Respective times, in hours, after deposition on washed agar: 10.8, 11.25, 11.7, 11.9, 12.4, 13.0, 14.4.

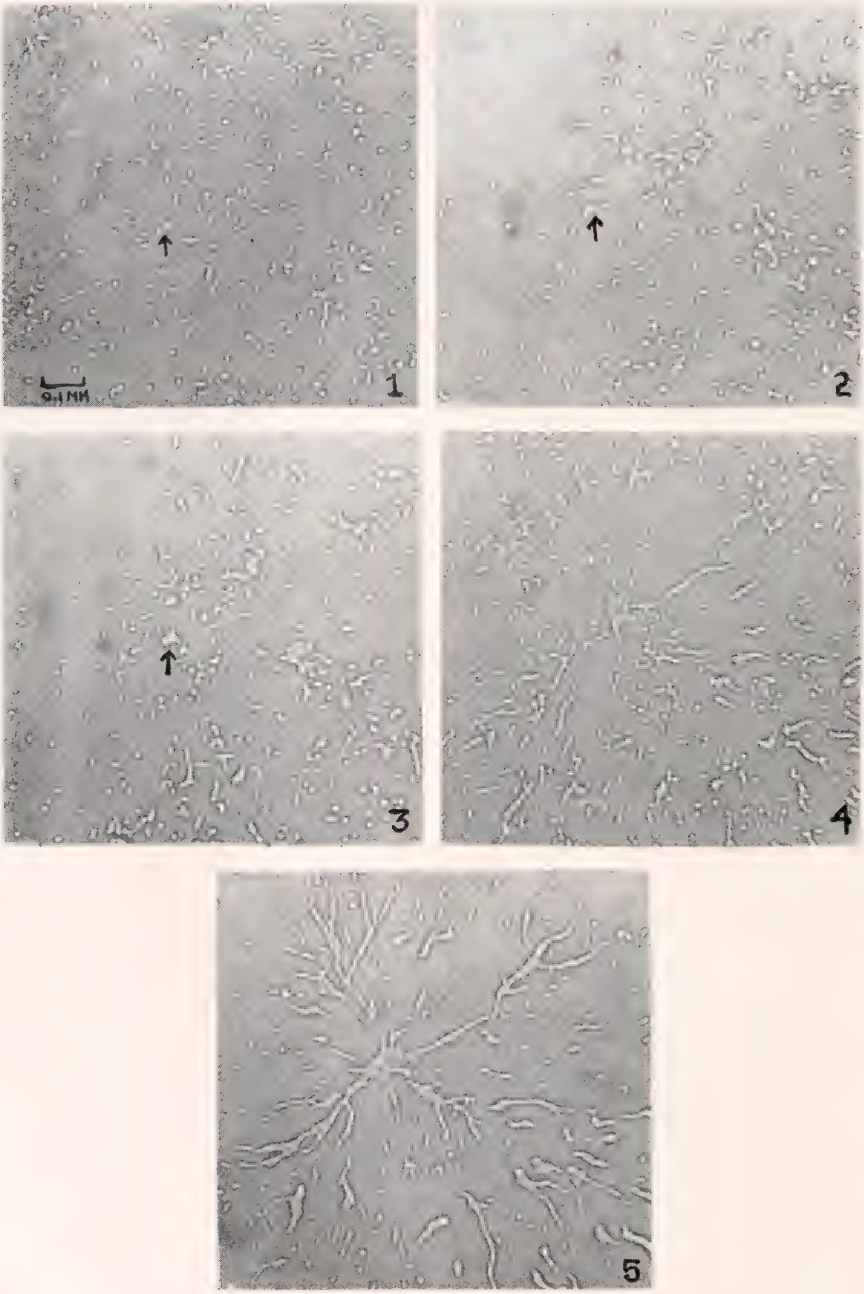

FIGURE 3. Time lapse photomicrographs of an aggregation. The arrows point to the I-cell. In photograph No. 2, the I-cell was joined by a few neighboring R-cells to form a tiny central clump. Photograph No. 3 was the last clearly discernible position of the I-cell.

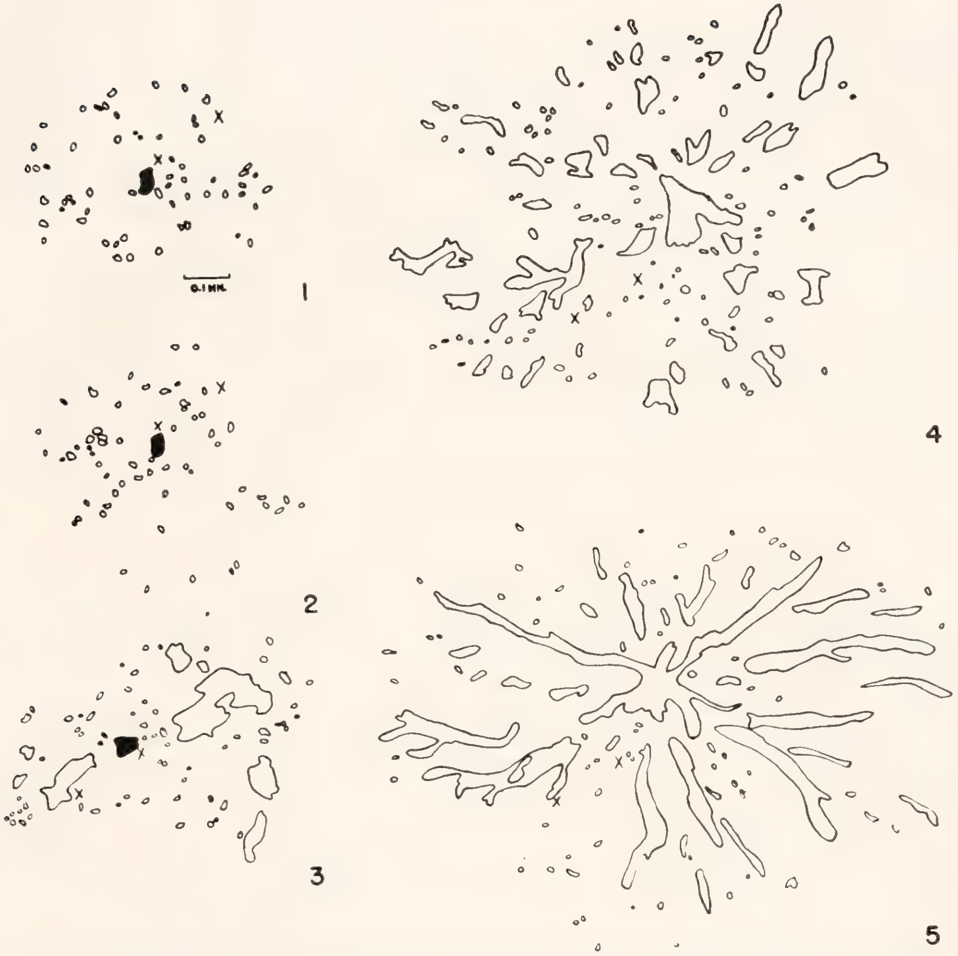

FIGURE 4. Time lapse camera lucida drawings of an aggregation. See legend of Figure 2 for details. Respective times, in hours after deposition on washed agar: 12, 12.5, 13, 13.4, 14.

nestle against it. This occurred in all but a few of the aggregations studied. The last clearly discerned position of the I-cell is in Figure 2-D. Its position was barely visible in Figure 2-E as the I-cell enlarged and extended to the right. Meanwhile, the previously formed clumps enlarged and new ones appeared concentrically about and at progressively greater distances from the I-cell. At this time, the loose cells and those in the clumps elongated and oriented radially. This caused the clumps to attain the appearance of streams. The position of the aggregative center then emerged clearly (Fig. 2-F) and is seen to have occupied the last known position of the I-cell. Ultimately the streams moved into and joined the center, producing the usual conical cell mass. Figure 3 is a series of photomicrographs of another aggregation in which the center again formed at the I-cell.

A typical sequence in which a center was established near the I-cell is shown in Figure 4. Again, the first sign of impending aggregation was the appearance of cell clumps, although on this occasion, no clump formed around the I-cell. A particularly large clump appeared at a distance of about 200 μ from the I-cell (Fig. 4-C). The I-cell then moved into a small clump immediately above the upper right reference mark. The cells elongated and oriented radially and a center was established at a distance of about 100 μ from the last known position of the I-cell. Thus the only real difference between the sequences shown in Figure 2 and Figure 4 is the appearance of the abnormally large clump, and this event always preceded the establishment of a center near, rather than at, the I-cell. In 10 of 19 cases, the R-cells entered the aggregate but the I-cell remained outside. In the other 9 cases the I-cell was also swept into the aggregate.

Figure 5 shows that a center need not form at a point along the previous path of the I-cell. The four cases were chosen because the respective I-cells wandered along relatively straight paths and could therefore clearly illustrate that such a relation did not exist. Cases 3 and 4 are particularly pertinent in that the centers did not form at the I-cells but did so at distances of 250 and 300 μ, respectively.

B. Aggregation after I-cell removal

The fact that I-cell removal can prevent subsequent aggregation was established in previously reported experiments (Ennis and Sussman, 1958b). They also showed that, to be effective, the removal must be accomplished at a very early stage of the pre-aggregative period. Thus, I-cells were removed from drops containing 500 myxamoebae within two minutes after they had been dispensed on washed agar. The incidence of centers 16 hours later (at which time all aggregations were completed), was only 9% of the incidence in the control drops from which I-cells had not been removed. If, however, the I-cells were permitted to remain for about 5 minutes before removal, the incidence of centers rose to 40% of the control value. Removal at 20 minutes increased the incidence to 67% of the controls and removal at one hour was totally ineffective, *i.e.,* equal percentages of aggregates developed in the controls and in populations from which I-cells were removed. Clearly, then, the presence of the I-cell in the immediate vicinity of the R-cells, even for a few minutes, is sufficient to produce an inductive effect.

Experiments performed since then have indicated that the I-cells can exert this inductive effect upon R-cells well outside of their immediate vicinity, albeit they require more time to do so. Replicate samples of 6000 washed myxamoebae were dispensed on washed agar at the optimal density of 200 cells/mm.^{2}. The excess fluid was absorbed by the agar and after one hour's incubation, an area, 1 mm.^{2}, was delineated at the center of each drop by scoring the agar surface lightly with two pieces of razor blade, mounted parallel at a distance of 1 mm. The cells outside of the square were brushed away, thereby leaving replicate samples of 200 myxamoebae at a density of 200 cells/mm.^{2}.

Since the distribution of I-cells has been found to be 1:1940, one would expect about 10% of the squares to have contained I-cells and accordingly to have aggregated. As may be seen in Table I, precisely 10% of the squares so treated did aggregate. Thus, it can be said that all of the aggregates observed must have been contributed by those squares that contained I-cells and that no I-cells lying

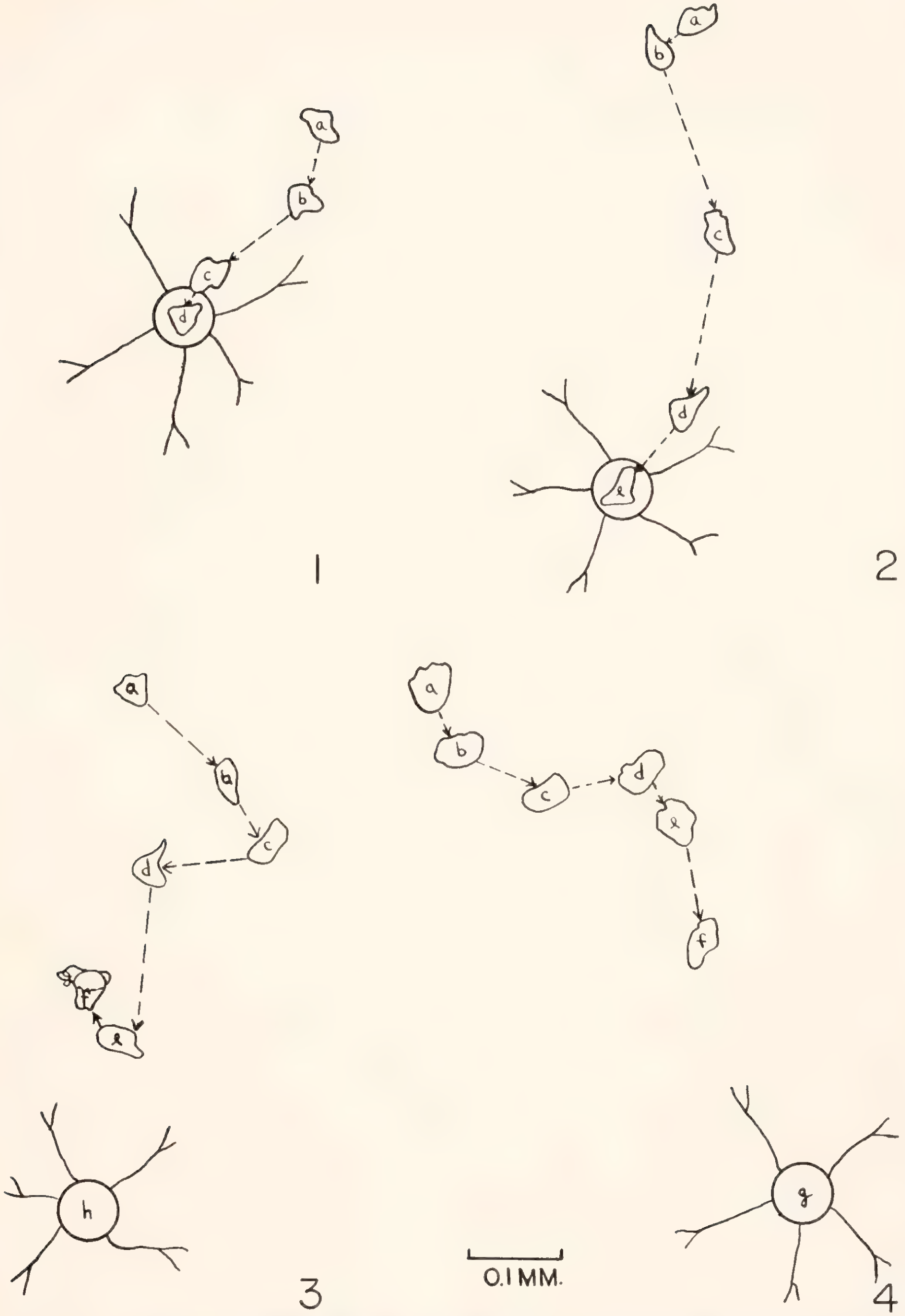

FIGURE 5. Relation between aggregative centers and previous migratory pathways of I-cells in four aggregations. In the top two, the centers coincided with the final positions of the I-cells. In the bottom two, they did not. The respective times, in hours, at which the first and last camera lucida drawings were made after deposition on washed agar: 10.8, 11.9; 9.8, 12.25; 9.8, 14.0; 10.0, 13.0.

TABLE I

After the stated periods of pre-incubation, squares were scored on the agar surface and outlying cells were brushed away. See text for details.

Pre-incubation period in hours	No. of squares	No. with aggregates	%
1	120	12	10.0
4–5	120	20	16.7
6–8	120	59	49.0
10–12	120	109	91.0

outside the boundaries of said squares for the one-hour period and then brushed away, appear to have exerted an inductive effect upon the R-cells lying within. When, however, a period of 4–5 hours elapsed before the squares were delineated and the outlying cells removed, the incidence of aggregates within the square rose to 16.7%. After 6–8 hours the incidence was 49% and after 10–12 hours, 91%. (At 12 hours, but not at 10, the cells were elongated and were beginning aggregation.) Thus, even at 4–5 hours, significantly more aggregates appeared than could be accounted for simply by the presence of I-cells within the squares. The data would therefore seem to force the conclusion that the I-cells lying outside the squares must have exerted an inductive effect upon the R-cells within. It is important to note that, since the total cell density was 200 cells/mm.^{2}, the density of the I-cells would have been about 1 per 10 mm.^{2}. Thus the I-cells in exerting their effect acted over truly fantastic distances.

The objection may be raised that not only the outlying I-cells were removed but also the outlying R-cells. Why, then, could one ascribe the inductive effect to the latter? The answer lies in the fact that when replicate samples of from 250 to 2000 myxamoebae were dispensed at densities even greater than 200 cells/mm.^{2}, a Poisson distribution of aggregates was obtained in strict accordance with the distribution of I-cells. If R-cells had any inductive capacity of their own at these densities, why then did not every sample aggregate regardless of whether an I-cell was present or not?

In summary, the data indicate that the I-cell performs its mission at the early stages of the pre-aggregative period. The immediate neighbors of the I-cell require its presence for only a few minutes and can then subsequently aggregate in its absence. The more remote neighbors can also be affected if the I-cell is allowed to remain for a longer period of time. It is difficult to explain these results save by the assumption of a diffusible "initiator substance."

C. *The initiative capacity of R-cells*

As mentioned previously, when I-cells were micromanipulated to test areas, they could induce the test populations to aggregate, whereas R-cells at the same stage of development could not (Ennis and Sussman, 1958b). In these experiments, the I-cells and R-cells were micromanipulated to the test areas within 20 minutes after they had been dispensed on washed agar. The question arose as to whether or not R-cells which had been incubated for periods longer than 20 minutes prior to micromanipulation might not display initiative capacity.

TABLE II

After the stated periods of pre-incubation, R-cells were individually micromanipulated to test areas. See text for details

Pre-incubation period in hours	Experimental			Background		
	Total	No. with aggregates	%	Total	No. with aggregates	%
1	53	7	13.2	79	11	13.9
4–6	65	14	21.6	250	29	11.6
10–12	71	26	36.6	70	9	12.8

Washed myxamoebae were dispensed on washed agar at a density of 150–200 cells/mm.^{2}. After 1, 4–6, and 10–12 hours, R-cells were picked up individually with a glass loop mounted in a deFonbrune micromanipulator and moved to test areas. The test areas had been prepared by dispensing washed myxamoebae on washed agar at a density of 250 cells/mm.^{2}, one hour prior to use. After the excess fluid had been absorbed, an area, 1 mm.^{2}, was delineated in the middle of each drop as described in the previous section. The outlying cells were brushed away leaving test squares containing 250 myxamoebae at a density of 250. The center:cell ratio being 1:2200, one would expect 11.3% of the squares to have aggregated spontaneously. The background controls shown in Tables II and III showed an incidence of 72 squares with aggregates out of a total of 578, or 12.4%. The extent to which addition of R-cells, pre-incubated for periods between 1 and 12 hours, affected the background incidence is shown in Table II. R-cells pre-

TABLE III

Initiative capacity of R-cells tested upon their developmental juniors

A. Samples with I-cells			Samples without I-cells		
No.	No. with aggregates	%	No.	No. with aggregates	%
21	18	86	13	0	0

B. Experiment	R-cells from samples with I-cells			R-cells from samples without I-cells			Background		
	Total	No. with aggregates	%	Total	No. with aggregates	%	Total	No. with aggregates	%
A	27	5	18.5	27	8	29.6	54	8	14.8
B	27	8	29.6	27	3	11.1	53	8	15.1
C	36	8	22.2	30	7	23.3	72	7	9.7
Total	90	21	23.4	84	18	21.4	179	23	12.8

A. Samples of 500 cells were dispensed on washed agar. Twenty-one which certainly contained I-cells and 13 which certainly did not were chosen. The percentages of samples that produced aggregates are shown.

B. After 8 hours' pre-incubation, R-cells, taken from the samples with and without I-cells, were micromanipulated to test areas. See text for details.

incubated for one hour did not affect the background frequency but increases of 10 and 24% over background were obtained by adding R-cells pre-incubated for 4–6 and 10–12 hours, respectively. In other words, when pre-incubated for 10–12 hours and then moved to test areas, one out of four R-cells could induce the formation of a center among the test cells, 12 hours after its introduction.

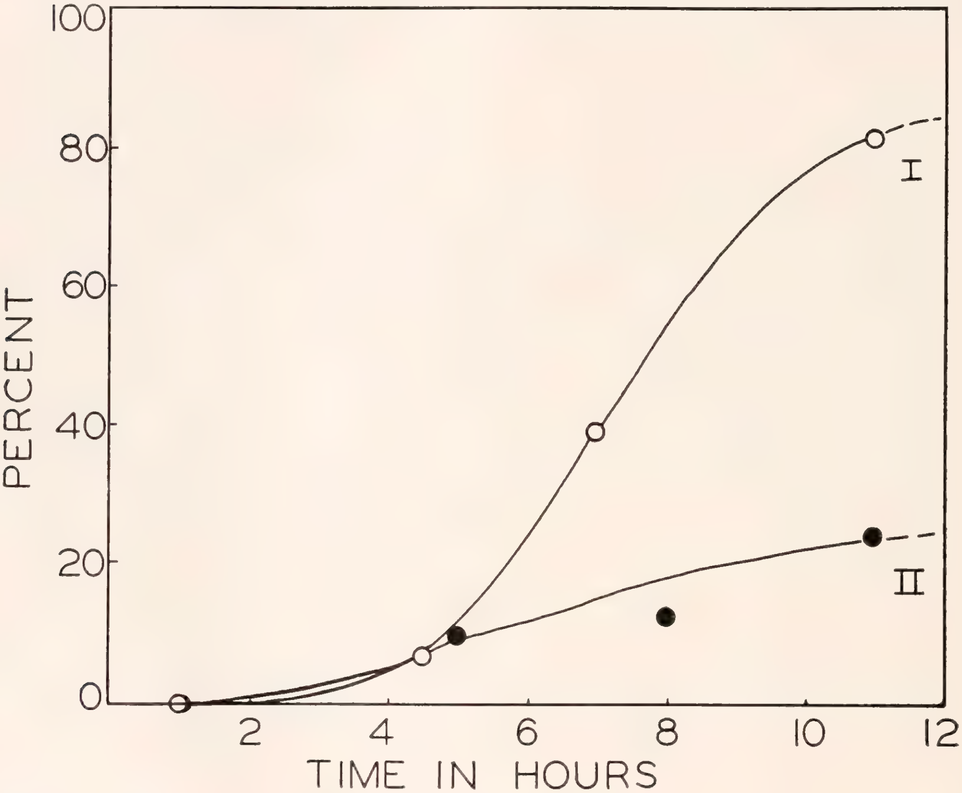

Figure 6. A kinetic comparison of: I. The capacity of small population samples to aggregate when isolated from their neighbors after varying periods of incubation. Ordinate: per cent of 250 cell samples that aggregated. Abscissa: time of incubation on washed agar prior to isolation. (Data from Table I.) II. The capacity of R-cells incubated for varying times on washed agar to initiate centers amongst their developmental juniors. Ordinate: per cent of R-cells capable of initiation. Abscissa time of incubation on washed agar prior to their micromanipulation to test areas. (Data from Table II.)

Figure 6 is a graphic comparison of the kinetics of induction of centers in test squares (I) by progressively delayed removal of outlying I-cells (data from Table I) and (II) by addition of pre-incubated R-cells (data from Table II). The crude kinetic similarity suggested that the outlying I-cell might not only be responsible for the subsequent aggregation of the R-cells but also for the concomitant increase in their capacity to themselves initiate centers. To test this possibility, replicate samples of 500 washed myxamoebae were dispensed on washed agar. In three experiments 21 samples were chosen which certainly contained I-cells and 13 which certainly did not. The data in Table III confirm the correctness of these choices since 86% of the samples said to contain I-cells aggregated while none of those said not to contain I-cells did so. After these samples had been incubated for 8 hours, R-cells were picked and moved to test squares as described in the preceding paragraph. Table III shows that R-cells, whether pre-incubated in the presence or absence of I-cells, were equally capable of inducing center formation. Thus, the rise of the initiative capacity of the R-cells during the pre-aggregative period is not dependent upon their contiguity with I-cells. Two points must be kept in mind here. First, it must be remembered that prior to their deposition on the washed agar, R-cells had all been in contact with I-cells and therefore could have been at this time the subject of interactions emanating from the latter. Second, even though the R-cells after 12 hours of incubation had attained a significant degree of initiative capacity, they fell far short of the level displayed by the I-cells after only 20 minutes of incubation. Therefore, the phenotypic difference between the two cell types in this respect remains clear.

Finally, the results reveal a most puzzling paradox. When R-cells were pre-incubated for 8 hours in the absence of an I-cell and then placed in the presence of test cells for an additional 12 hours, at least one out of ten could induce center formation. Yet the samples from which these R-cells originally came, when incubated for a total of 20 or indeed 36 hours, had not aggregated. It is clear, therefore, that the observed increase in the initiative capacity of R-cells during the pre-aggregative period in the development of a population is of no consequence to the ultimate aggregation of *that* population. In other words, the initiative capacity of such R-cells, demonstrated by movement to another population, is an experimental artifact bearing no relation to normal aggregation but which may possibly be used to understand the biochemical and genetic differences between the I-cell and R-cell *Phenotypes.*

DISCUSSION

The data presented here and previously suggest a developmental program of slime mold aggregation that may serve as a useful working hypothesis.

I-cells arise during the growth of an R-cell population (which in turn had originated from the spores of the preceding fruit), and attain a steady-state ratio of approximately 1:2000 early in the exponential phase (Sussman, 1956; unpublished data). Entrance into the stationary phase marks the beginning of the pre-aggregative period. At the beginning of this period, the I-cells secrete material which, during the ensuing 12 hours, so conditions the neighboring R-cells as to induce them to aggregate. This interaction, as might be expected, affects the nearest neighbors first but its influence is progressively extended. Concomitant with, but unrelated to either the presence of the I-cell or the subsequent course of aggregation in the same population is a significant rise in the initiative capacity of the R-cells themselves. Such cells upon extended incubation never do attain the degree of initiative capacity displayed by the I-cells nor can they act upon their developmental contemporaries but only upon cells at an earlier developmental stage to which they have been added by the observer.

The first overt sign of aggregation is the formation of cell clumps concentrically about and usually at the I-cell. This is followed by excitation and elongation of the loose and clumped cells in response to the chemotactic complex (Sussman *et al.*, 1956; Shaffer, 1956; Sussman, 1958). The appearance of oriented streams establishes the position of the aggregative center. This is usually coincident with the final position of the I-cell but sometimes with the position of a particularly large clump nearby, and possibly reflects the point of greatest production of the chemotactic complex. In the latter case, the position of the center need bear no relation to the previous path of the I-cell.

The picture as drawn raises many questions and offers a number of predictions under current study. The most important of the latter involves the hypothetical existence of an "initiator" substance. In view of the I-cell removal experiments, one ought under the same conditions to be able to induce test cells to aggregate by dispensing them in an area previously but no longer occupied by an I-cell. This is being tested. The I-cell addition experiments raise the question as to what is the minimum period of time after contact with the I-cell in which the induced R-cells can begin aggregation. Is the 12-hour period subsequent to contact mandatory or does it involve preparations by the R-cells for aggregation, unconnected with the function of the I-cell? In the latter case, one ought to be able to preincubate the test cells for twelve hours, add I-cells, and observe the onset of aggregation very shortly thereafter.

The fact that R-cells can also attain initiative capacity to a far smaller degree, albeit much later than do the I-cells and ineffectively so far as inducing their contemporaries to aggregate is concerned, still suggests that the metabolic pathways involved in initiation are not unique to the I-cells. Indeed, one may imagine that the sole basis for the difference between I-cells and R-cells in this respect is the much greater size of the former. Perhaps, then, any of the diverse methods for producing giant cells may serve to create initiators just as does the normally occurring R-cell to I-cell transformation. This point is also under current study.

Summary

Dictyostelium discoideum myxamoebae occur as two distinct morphological types, termed I-cells and R-cells. Data presented in a previous publication demonstrate that I-cells can initiate centers of aggregation and suggest compellingly that they are in fact the initiator cells for normal aggregation. The present communication extends and amplifies these findings.

A. Time lapse camera lucida drawings and photomicrographs illustrate the sequence of events during the onset of aggregation.

B. Small population samples of myxamoebae, when isolated from their neighbors shortly after deposition on washed agar, showed a distribution of aggregative centers consistent with the distribution of I-cells within the samples. Longer periods of contact with neighboring cells (including other I-cells) that surrounded the samples prior to isolation permitted progressively greater proportions of the samples to aggregate. The possibility arises of an "initiator substance" whose effect may extend over relatively great distances.

C. R-cells, incubated for long periods of time on washed agar, were found to have acquired initiative capacity. At best, only a small proportion did so and fur-

thermore could only induce the formation of aggregative centers amongst their developmental juniors (by twelve hours) but not amongst their developmental contemporaries.

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SHELL REPAIR IN CHITONS

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Cryptochiton stelleri (the "gumboot") is not only the largest member of the class Amphineura, but also one of the most specialized in that the girdle tissue has completely overgrown the skeletal plates (Heath, 1897). It therefore lacks the outer shell layer, the tegmentum. While preparing some of the skeletal plates for display, it was noticed that occasional plates were cracked and that many of these cracks were repaired by an amber-colored membrane resembling conchiolin. It seemed of interest to determine the frequency of damage, the stages of repair, the possible significance of this ability to the survival of the animal, and the relative incidence of breakage and repair in several other species of chitons (*Katherina tunicata, Mopalia hindsii*).

Cryptochiton (*Amicula*) *stelleri* is a subtidal browsing herbivore, but it is also found in fair numbers up into the middle zone of the intertidal region. When found in the intertidal zone it is attached loosely to rock encrusted with coralline algae or to algal curtains, and occasionally it is found on a sandy bottom. *Cryptochiton* holds to its substrate only gently and can be removed easily by hand. It is also dislodged by wave action as evidenced by the large number (approximately 75) counted on three local beaches after a heavy storm in April, 1958. The plates of the storm-tossed animals were shattered and all but five of the animals were dead. It is possible that after seeking food in shallower tidepools and crevices during high water, the chiton is left by the subsequent receding tide and falls from its loosely-held position among the algae. Caught by wave action, it may be beaten against the rocks before it can re-establish its hold or before it can get back to deeper waters. The animals when strongly stimulated in the laboratory have been seen to contract with sufficient force to crack their plates; perhaps some are also broken in this manner in nature.

Of the 146 sets of plates (Fig. 1A) collected [2] 87, or 59.5 per cent, had one or more plates broken (about 18 per cent had one, 17 per cent had two, 11 per cent had three, 6.2 per cent had four, 3.4 per cent had five, 2.7 per cent had six, 1.3 per cent had seven, but none had all eight). Two animals had seven of the eight plates broken. The middle plates were broken most often, these being the widest and flattest (6.2 per cent plate 1, 11.4 per cent plate 2, 14.1 per cent plate 3, 18 per cent plate 4, 21.2 per cent plate 5, 16.8 per cent plate 6, 8.4 per cent plate 7, and 3.9 per cent plate 8). Often two or three adjacent plates were found with similar breaks, suggesting a blow from a large surface.

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[2] The chitons were being used in a study of the annual reproductive cycle and of the biochemistry of the blood and tissues; hence the plates were available in numbers, from specimens collected for these purposes.

Plates that had been broken just prior to the animal's death, either by storms or in the laboratory, showed a clean cleavage with the parts fitting perfectly together. Depending upon the severity of the blow, the cleavage was in a single straight line or in an arborescent pattern. Repairs were seen in few plates that had been shattered into as many as seven pieces.

The first step in repair is the formation of a strip of membrane overlapping the crack on both sides of the plate. The second stage seems to be the accumulation of fine granules of a calcium salt, presumably in the form of carbonate (Bevelander and Benzer, 1948), under the conchiolin strip with the concomitant erosion of the underlying crystalline shell. This erosion often extends for some distance laterally from the crack under an extension of the membrane strip (Fig. 1C). The last step in repair (Figs. 1D–G) is the invasion or growth of existing minute crystals (Bevelander and Benzer, 1948; Bevelander, 1953) of the surface of the membrane strip by crystals of calcium carbonate in the form of aragonite (Prenant, 1927). The crystals are imbedded in the surface of the conchiolin, leaving a ridge over the crack, and often an air space or a layer of membrane between the old shell and the new material (Fig. 1E). This leaves the plate weakened so that a second blow usually splits the plate along the old crack.

To determine the rate of repair of broken plates, five chitons subjected to hammer blows were kept in the laboratory with ample food and in running sea water, and sacrificed after varying lengths of time. The results are quite variable but they serve to illustrate the slowness of repair. For example, while one chiton developed membranes around the cracks in twenty days and granular calcium carbonate deposition in twenty-four, another showed no visible sign of repair in the same period of time. In still another chiton, dissected sixty days after breaking the plates, crystalline calcium carbonate was evident in the cracks. However, in two chitons examined 100 days after injury, only membranes had been laid down over the cracked edges of the plates.

Energy for mobilization of the shell calcium is available only during active feeding and digestion in some mollusks (Wagge, 1951, 1952; Robertson, 1941). The effects of starvation were not tested here in view of the variability of results with well-fed specimens.

Wilbur and Jodrey (1955) inhibited shell deposition in the oyster with carbonic anhydrase inhibitor. However, no tests were made with such inhibitors on *Cryptochiton* in view of the variability of results and the long time required for repair of broken skeletal plates. Furthermore, it is not even known whether amphineurans possess carbonic anhydrase although Freeman and Wilbur (1948) found it in most, but not all, of the species of gastropods and pelecypods tested.

Katherina tunicata

Fifty-five sets of plates of *Katherina* were examined and only five plates were found to be broken, although many of them were eroded to some extent, possibly by a disease. Of these, two showed slight evidence of repair. One had a thin membrane with a few lime crystals, the other showed an old crack completely repaired. It is possible that the other cracked shells were broken when the eviscerated specimens were boiled to loosen the plates for examination. One valve broken experimentally showed a conchiolin membrane after a few weeks.

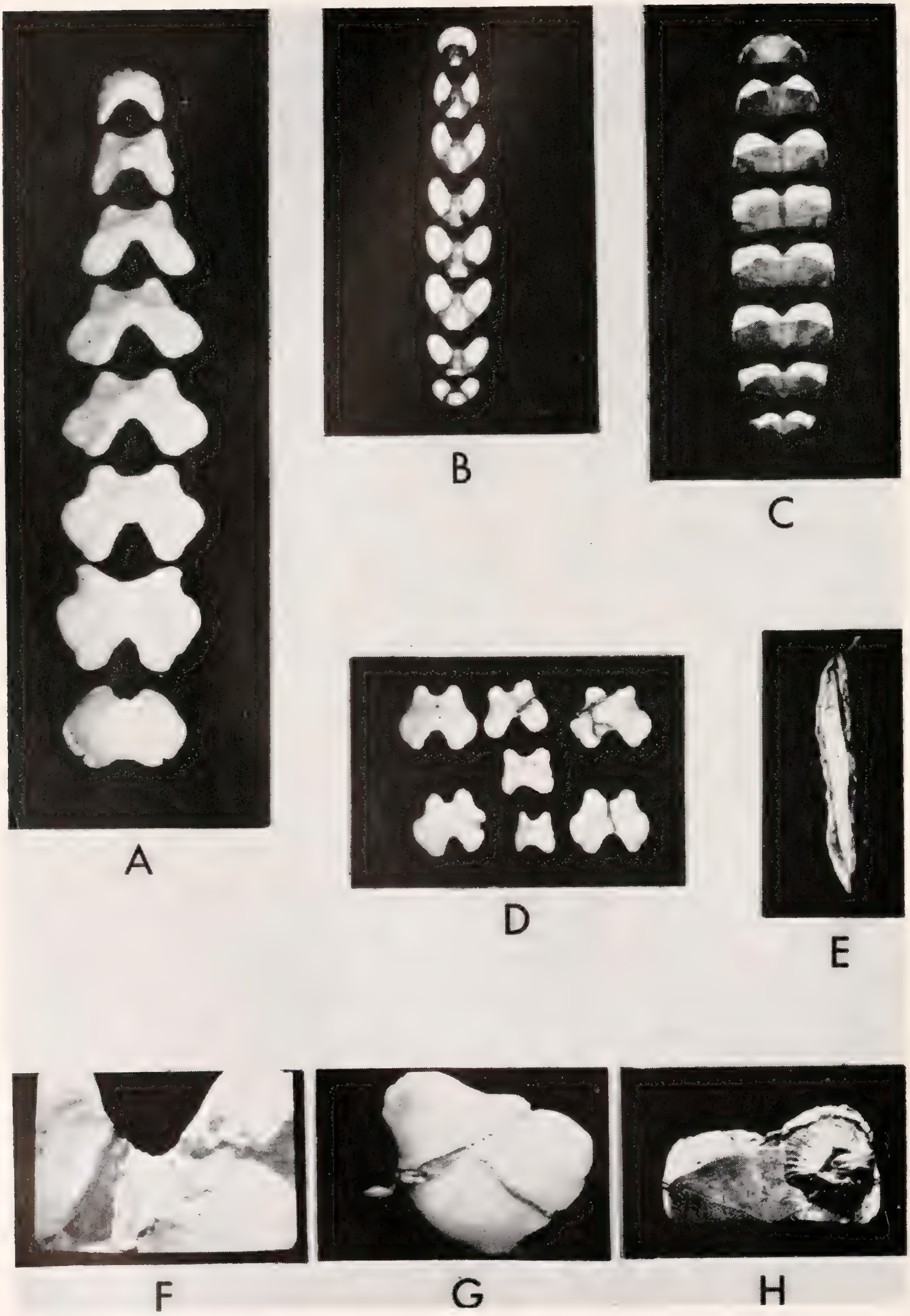

FIGURE 1.

Katherina lives in the surf zone on exposed shores among the sea palms and between mussel beds where, at certain times of the day, it withstands an almost continual pounding by the waves. Even at low tides the animals hold fast to bare rock or crustose algae with such strength that a knife or screwdriver is needed to pry them loose. At that, an inexperienced collector will often get only the plates and girdle, the foot and viscera remaining on the substrate. The storms of 1958 which left so many specimens of *Cryptochiton* on the beaches presumably failed to dislodge specimens of *Katherina;* at least none was seen on the beaches with *Cryptochiton.*

The infrequency of broken plates in *Katherina* suggests that its plates are proportionally stronger than those of *Cryptochiton.* The average weight of the eight plates (10 specimens; average wet weight 33 grams) was 19.6 per cent of the wet weight of the entire chiton, in comparison to the 7.4 per cent for *Cryptochiton* (45 specimens; average wet weight 850 grams).

It is also possible that the shape of a skeletal plate has some bearing on its resistance to shock. Plates No. 2 to No. 7 of *Cryptochiton* are in the shape of butterflies (Fig. 1A) and are relatively flat. The skeletal plates in *Katherina* consist of a heavy, roughly circular, disc with one pair of thin lateral lobes (Fig. 1B).

Mopalia hindsii

Twenty-six sets of skeletal plates of *Mopalia* were examined and, other than chipping along the edges of the thin membrane, eleven had broken plates (six had one plate broken, three had two and one each had three or four plates broken). The most common crack was from the lateral notch to the beak of the plate. Along this line the plate is porous. Although many of the cracks are clean and may have resulted from boiling eviscerated specimens to release the plates, *Mopalia* suffers a fairly high incidence of infection from an unidentified boring animal which weakens the prismatic layer of the plate with long tunnels. One of these weakened plates was broken and the shell was thickened along the cracked tunnel. A thin membrane and some lime crystals were also deposited after a lapse of several weeks along cracks in plates No. 2 to No. 7 broken by a blow from a hammer. One unbroken plate showed deposition of new material where an attached barnacle overlapped the edge of the plate (Fig. 1H). *Mopalia* therefore can to some extent repair its skeletal plates.

The specimens of *Mopalia* used in this work were collected from concrete pilings in Monterey Harbor, a relatively protected habitat. While the skeletal plates are broad and flat (Fig. 1C) and in this respect resemble those of *Cryptochiton,* at the same time they constitute about 22.1 per cent of the wet weight of the animals. Apparently they are adequate for the conditions to which the animals are exposed.

FIGURE 1. A. Shell plates of *Cryptochiton stelleri.* × ½. Plate No. 1 (anterior) is toward the top of the page. B. Shell plates of *Katherina tunicata.* × ½. Plate No. 1 (anterior) is toward the top of the page. C. Shell plates of *Mopalia hindsii.* × ⅔. Plate No. 1 (anterior) is toward the top of the page. D. Representative cracks in shell plates of *Cryptochiton* undergoing repair. × 1/10. E. A section along a crack in a plate of *Cryptochiton* showing the space between the old shell and the new material deposited during repair. × 1. F. Lateral extension of the conchiolin strip (dark material) over two breaks. × 1. G. Invasion of the conchiolin strip (dark) by crystalline calcium carbonate (light). × 1. H. Deposition of new shell (above, right) along edge of barnacle attached to *Mopalia plate.* × 1.

Summary and Conclusions

The skeletal plates of *Katherina tunicata* and *Mopalia hindsii* are sturdy, constituting about a fifth of the wet weight of the animal. They were seldom found broken in the specimens examined, but some broken plates were undergoing repair. The skeletal plates of *Cryptochiton stelleri,* on the other hand, are flat and thin and constitute only 7.4 per cent of the wet weight of the animal. The majority of cryptochitons examined showed breaks in one or more skeletal plates and in almost all of these, some degree of repair and deposition of membrane or mineral could be observed. The ability to repair its plates is probably of value to this species in view of the weakness in design of its skeleton.

Irregularities of plates and variations in numbers of skeletal plates have been described for other species of chitons (Crozier, 1919; Berry, 1925, 1935; Taki, 1932). It is interesting that apart from an occasional asymmetrical terminal plate of a *Cryptochiton,* no such irregularities in number or shape were observed in the three species of chiton studied here.

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THE JUVENILE HORMONE. I. ENDOCRINE ACTIVITY OF THE CORPORA ALLATA OF THE ADULT CECROPIA SILKWORM

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The endocrine role of the corpora allata of insects was discovered by V. B. Wigglesworth (1934, 1936) over twenty years ago. In a series of simple and decisive experiments on *Rhodnius* he showed that the corpora allata secrete a "juvenile hormone" which opposes metamorphosis. In these early studies Wigglesworth also recognized that the corpora allata undergo pronounced changes in endocrine activity during the course of metamorphosis; namely, that they are active in the immature nymph, inactive in the mature nymph just prior to metamorphosis, and active again in the adult insect after metamorphosis. Subsequently, the general validity of these conclusions has been confirmed repeatedly and found to apply to both hemi- and holometabolous insects (for review, see Wigglesworth, 1954, pages 56–64).

During the past twelve years, in the course of studies of the metamorphosis of the Cecropia silkworm, the juvenile hormone has necessarily been an object of detailed attention. While confirming the essential elements in Wigglesworth's theory, the study has helped to resolve certain persistent mysteries and, more recently, has pointed the way to the successful extraction and purification of the hormone itself. This first of a series of communications is concerned with the endocrine activity of the corpora allata of the adult moth.

MATERIALS AND METHODS

1. *Experimental animals*

The experiments were performed on Cecropia, Cynthia, and Polyphemus silkworms. Taxonomists continue to amuse themselves by changing the generic and specific names of these Saturniids. What began as *Phalaena cecropia* became *Samia cecropia*, then *Platysamia cecropia*, and now *Hyalophora cecropia* (Michener, 1952). The Cynthia silkworm, known throughout the world as *Philosamia cynthia*, was changed to *Samia walkeri*, and then back to *Samia cynthia*. *Telea polyphemus* is now *Antheraea polyphemus*. As in the analogous cases discussed by Wald (1952, page 339), the "common names" have escaped the attention of taxonomists and have remained firm and unchanging. Therefore, the common names will be used routinely in the present reports.

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[1] This study was aided by a grant from the National Institutes of Health of the U. S. Public Health Service. It is a pleasure to acknowledge the advice and counsel of Prof. Berta Scharrer.

Cecropia silkworms were reared under nylon nets on wild-cherry trees. Polyphemus were reared on oak or maple; Cynthia, on cherry or ailanthus or purchased from dealers. The cocoons were harvested and stored as previously described (Williams, 1946a; Shappirio and Williams, 1957).

2. *Surgical procedures*

Experimental animals must be deeply anesthetized during surgical procedures. We use carbon dioxide for this purpose and with mixtures of air and carbon dioxide have maintained pupae anesthetized for as long as one month without injury. Groups of animals are placed in a capped, flat-bottom Büchner funnel and exposed for about twenty minutes to a slow stream of carbon dioxide from a compressed cylinder. The gas is bubbled through water *en route* to the funnel. The animals are flaccid when fully anesthetized, and one can no longer elicit any movements of the abdominal segments.

Surgical procedures are performed in a second Büchner funnel (diameter 11 cm., height 3 cm.) which is mounted flush on the top of the operating bench. A slow stream of carbon dioxide is bubbled through water and passed through the bottom of the uncovered funnel. Carbon dioxide, being heavier than air, fills the cavity of the funnel and maintains a continuous anesthesia during the surgical procedure (Williams, 1946b).

Operations are carried out under the low magnification of the dissecting microscope, making use of 9 × oculars and 0.7, 1, or 2 × objectives. The foot of the microscope is removed and the vertical pillar permanently attached to the operating bench on the distal side of the funnel. A hinged-arm permits the microscope to scan the entire diameter of the funnel. In order to leave both hands free, the microscope is equipped with a foot-focusing device (designed and built by Mr. Robert Chapman of the Harvard Biological Laboratories). Illumination is provided by a 6-volt microscope lamp (Zeiss "Osram") attached to and moving with the microscope. The lamp is equipped with an infra-red filter.

Anesthetized animals are transferred to the carbon dioxide-filled funnel for the surgical procedure. They are then returned to air, placed in individual numbered glass containers ("creamers"), and stored in a room having a controlled humidity of sixty per cent and a temperature of 25° C.

Dissecting instruments consist of the following: watchmaker's forceps (Dumont "rustless"; two of No. 3 and two of No. 5); a scalpel (Bard-Parker No. 3 handle with a No. 11 detachable blade); stainless iris scissors curved on the flat and closing to the tip; several forms of stainless steel iridectomy and micro-scissors; a stainless steel dental probe; a 5-ml. hypodermic syringe filled with insect Ringer and capped with a 25-gauge needle.

Prior to each group of operations the instruments are briefly rinsed in seventy per cent ethanol and wiped dry. Rigorous asepsis is unnecessary because the blood of the silkworms apparently contains an anti-bacterial substance that protects it from the ordinary contaminants. However, it fails to protect from insect pathogens and no diseased insect should be operated upon with the same instruments or even in the same room.

Healthy pupae can withstand almost any degree of surgery provided that a few crystals of the potent anti-tyrosinase, phenylthiourea, are placed in the operating field. We routinely use an equal part mixture of phenylthiourea (twice recrystallized from hot 95 per cent ethanol) and streptomycin sulphate, the two having been ground together in a mortar and stored in a capped vial in the refrigerator. Small amounts of the powder are removed and discarded within two days after being placed at room temperature.

Ephrussi-Beadle Ringer's solution is utilized containing 7.5 gm. NaCl, 0.35 gm. KCl, and 0.21 gm. $CaCl_2$, per liter of distilled water. The stock solution is brought to a boil, capped, and stored in the dark under refrigeration. Fungal contamination of physiological solutions, especially those containing bicarbonate, is a common source of difficulty when solutions are stored at room temperature.

Excised tissues and organs are transferred to small depression dishes made of black glass and filled with Ringer. Black plastic bottle-caps are also satisfactory for this purpose. Dissections of sacrificed animals are performed in a glass Petri dish which fits snugly into the cavity of the Büchner funnel. Plasticine is pressed into the bottom of the dish to receive short stainless steel pins. The dish is filled with Ringer and the dissection performed with the animal spread and pinned under the solution.

After surgical procedures on surviving pupae, Ringer's solution is added from a hypodermic syringe so that the blood is flush with the surface of the cuticle. The area of excised cuticle is then capped by a plastic window of appropriate size. The latter is punched or cut with scissors from cellulose acetate cover slips ("Turtox," thickness 1 or 2). The window is sealed in place with paraffin wax which is melted in an alcohol lamp and transferred with a curved needle or drawing pen. The melted wax adheres to the cuticle and the underside of the rim of the plastic slip provided that both are dry. The operating field is thereby equipped with a transparent window which permits one to look inside the living animal.

3. *Excision of pupal corpora allata and corpora cardiaca*

An anesthetized pupa is placed in a plasticine cradle in the bottom of the carbon dioxide-filled funnel. The cuticle of the facial region is first removed. For this purpose a scalpel incision is made through the integument on each side of the face. The two cuts are joined by a transverse cut and the rectangle of cuticle is grasped with forceps and pulled free from its attachment at the base of the legs. The insect's abdomen is then pressed forward with plasticine and held in this position so that the blood fills, but does not overflow, the operating field. The naked epidermis is grasped with forceps, split down the middle, and trimmed free with scissors. The brain is thereby exposed. This is pressed down in the field to reveal the tiny corpus allatum-corpus cardiacum complex on each side. The complexes are dorso-lateral to the brain and attached on each side to a large tracheal trunk at this position (see Figure 1). A pair of tiny nerves emerges from the posterior face of each brain hemisphere and passes to the corpus cardiacum on that side. These nerves are very delicate and difficult to see in a dissection of this type.

By means of forceps the connections between glandular complex and the adjacent trachea are broken, and the complex transferred to Ringer's solution in a black dish. Alternatively, the tracheal segment can be excised with iridectomy scissors and removed along with the glandular complex.

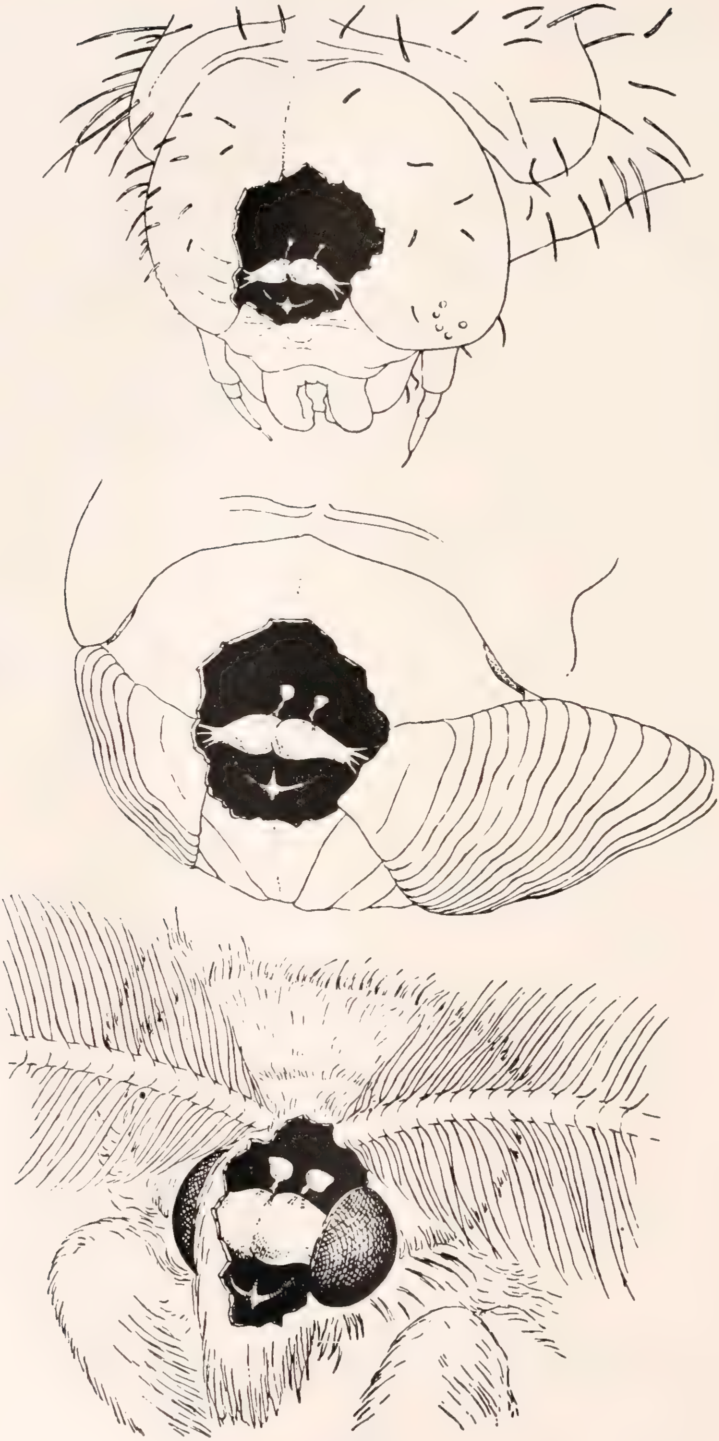

FIGURE 1.

4. *Excision of adult corpora allata*

The moth is anesthetized and its head dipped momentarily into seventy per cent ethanol to wet the scales and hairs. The head is then cut off with scissors and placed in Ringer's solution. (The headless moth will continue to live for approximately the normal life-span of 7 to 10 days at 25° C.)

The antennae are excised at their bases. Then with fine scissors the head is cut along the dorsal midline from its posterior margin to the mouth parts. The head is then spread apart with foreceps and pinned under Ringer. The pair of corpora allata-corpora cardiaca complexes is attached to the aorta just behind the brain. The brain is split in the midline to expose the aorta. The glandular complexes can now be broken free from the rear of the brain and transferred to a black dish by grasping the aorta with forceps.

Under the favorable conditions of illumination in the black dish, one can recognize the corpus cardiacum; it is attached by short nerves to the much larger corpus allatum. The latter is ordinarily flattened or wedge-shaped and subdivided into a number of lobes and lobules. If necessary, the glandular complex may now be subdivided into its constituent parts by breaking the nerves between corpus cardiacum and corpus allatum.

5. *Isolation of pupal abdomens*

This procedure has already been described for the Cecropia silkworm (Williams, 1947). The principal difficulty is to isolate the terminal abdominal segments without puncturing the fluid-filled midgut. This difficulty is circumvented by the use of the Cynthia silkworm. In this species the midgut contains only a solid, rod-like mass. Therefore the perforation of the midgut is inconsequential. The pupa is transected just behind the metathorax with a single transverse cut of a sharp razor blade. The abdomen is then supported with the cut surface facing upward. Crystals of the phenylthiourea-streptomycin mixture are spread in the wound, and Ringer's solution is added to fill the cavity of the abdomen. The wound is then capped with a plastic slip in which a central hole has been punched. The plastic is sealed in place with melted wax. Ringer is finally added *via* the central hole to replace all air, and the hole itself sealed with wax.

Results

1. *Role of the corpora allata in adult development and sexual maturation*

The pair of corpora allata-corpora cardiaca complexes was removed from each of a series of twenty chilled male or female Cecropia pupae *via* the facial approach. The integumentary defect was capped and sealed with a plastic window, and the animals placed at 25° C.

Adult development was initiated after about two weeks and proceeded in synchrony with the time-table for the normal development of Cecropia at 25° C. (Schneiderman and Williams, 1954). The moths, emerging after three weeks

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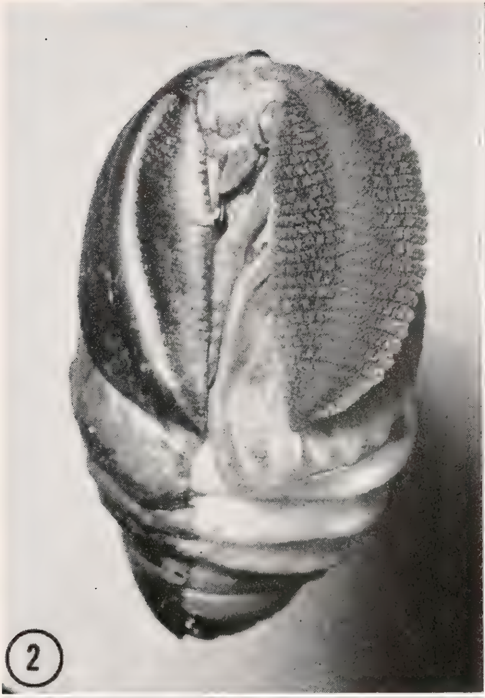
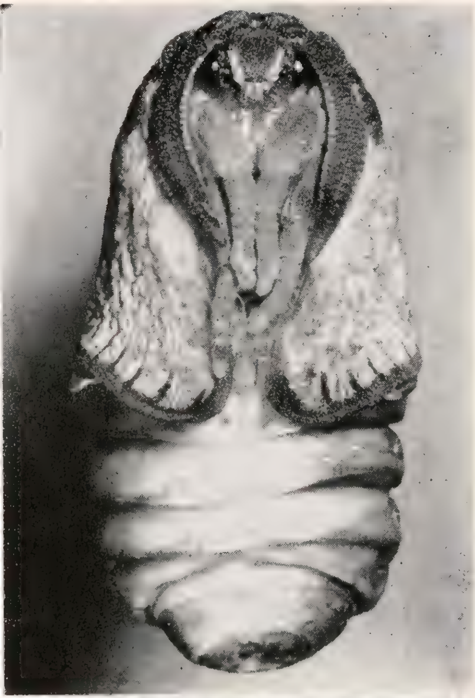
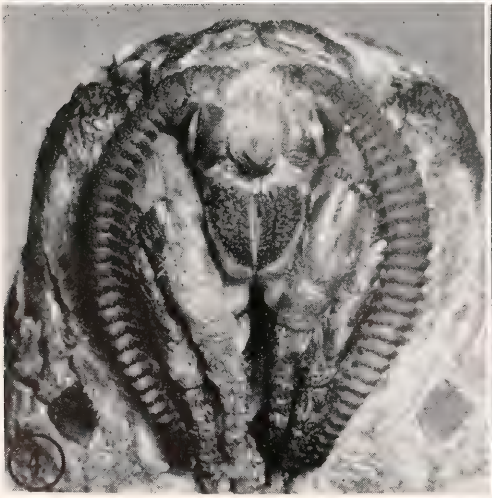
FIGURE 1. Brain and corpora allata of the Cecropia silkworm are shown in cutaway views of the head of larva (*top*), pupa (*middle*), and adult (*bottom*). The corpora allata are the two small bodies attached by tiny nerves to the back of the brain. (This figure is used with the permission of *Scientific American.*)







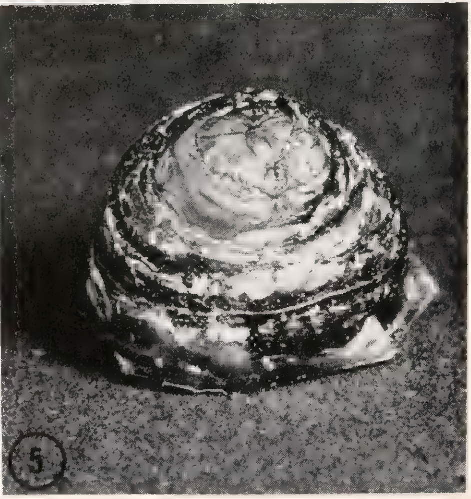


FIGURE 2. After receiving implants of three pairs of corpora allata of adult Cecropia, the Polyphemus pupa, here illustrated, has transformed into a second pupal stage. (See right side of preparation where the old pupal cuticle has been trimmed away.)

FIGURE 3. This Cecropia pupa received implants of two pairs of adult Cecropia corpora allata. Development has given rise to a mixture of pupa and adult. (The old pupal cuticle has been completely removed.)

of adult development, could not be distinguished from un-operated individuals. The females deposited a normal complement of eggs and both sexes survived for the customary period of 7 to 10 days at 25° C.

The absence of corpora allata was confirmed in dissections of many of these moths. All the internal organs, including the gonads, showed full and complete development. The abdomens of females were packed with ripe eggs, and the males showed normal spermatogenesis.

The experiment was repeated on a series of six male and six female pupae to produce moths lacking corpora allata. The two sexes were cross-mated and each of the six females was allowed to oviposit in a paper bag. A normal number (150–225) of eggs was collected from each female. These were placed under large nylon nets and the larvae reared to maturity on wild-cherry leaves. No deviation from normal development could be detected.

These experiments show that the corpora allata play no evident role in the transformation of the pupa into an adult Cecropia or in the gonadal function of the adult itself.

2. *Endocrine activity of adult corpora allata*

In the absence of any obvious function of the corpora allata of adult Cecropia, it is paradoxical to find that the glands, when excised and tested for endocrine activity, are more active in the moth than at any other stage in the life history (Williams, unpublished data). This fact was discovered eleven years ago in the course of an experiment performed for other purposes. It happened by chance that a pair of adult corpora allata was implanted into a brainless diapausing Cecropia pupa. Ten days later, the host showed the termination of diapause and the initiation of development. This result would have been puzzling in a normal diapausing pupa; in a brainless diapausing pupa it was incomprehensible.

Even more puzzling was the character of the development which then took place. Within two weeks the brainless pupa transformed, not into a moth, but into a bizarre creature in which large areas of pupal cuticle had been freshly formed (see Figs. 3 and 4). The animal, in short, was a mosaic of pupal and adult characteristics (Williams, 1952b).

During the past eleven years this result has been duplicated on numerous occasions. The experimental series includes fifty-one brainless Cecropia pupae which received one to three pairs of corpora allata-corpora cardiaca complexes derived from male or female Cecropia moths. As shown in Table I a total of twelve individuals (23 per cent) showed the result just described. The residual 77 per cent showed no effect of the implantation and continued to diapause. But the twelve positive experiments were of sufficient interest in themselves. Not only

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FIGURE 4. Pupal-adult monstrosity after implantation of adult corpora allata. Note the pupal cuticle on head, palps, and antennae. However, the wings show scale-covered adult cuticle and the eyes show considerable adult development.

FIGURE 5. This isolated pupal abdomen received implants of adult corpora allata, plus an injection of ecdysone. The tip of the old pupal cuticle has been torn away to reveal a second pupal abdomen that has formed.

Table I

Tests of adult corpora allata-corpora cardiaca complexes in brainless diapausing pupae*

Adult donors	Brainless hosts	Normal development	Mixed development	No development
Cecropia	Cecropia	0	12	39
Cecropia	Cynthia	0	2	2
Cecropia	Polyphemus	0	2	1
Cynthia	Cynthia	0	0	5
Cynthia	Cecropia	0	0	20
Polyphemus	Polyphemus	0	2	0
Polyphemus	Cecropia	0	4	10
Totals		0	22	77

* One to three pairs of complexes from male or female moths were implanted into each brainless pupa.

had the implants caused the formation of mixtures of pupa and adult; seemingly, they also had substituted for the brain and provoked the termination of diapause.

As shown in Table I, this result was duplicated when corpora allata of adult male or female Cecropia were implanted into brainless diapausing pupae of Cynthia or Polyphemus. Here again, a certain percentage of animals terminated diapause and developed into pupal-adult mixtures.

The corpora allata-corpora cardiaca complexes of male and female Cynthia and Polyphemus moths were also tested. The three species seem to differ among themselves in the endocrine activity of the adult corpora allata. For example, the corpora allata of adult Cynthia gave negative tests in all twenty-five preparations. By contrast, the glands of adult Polyphemus gave positive tests in six of sixteen preparations. Moreover, when used as recipients of implants, brainless Polyphemus pupae seemed to have a lower developmental threshold than the other two species, for four of five individuals gave a positive reaction to the implantation of adult corpora allata. In retrospect, Polyphemus appears to be the animal of choice for experiments of this type.

In the far more numerous tests of Cecropia corpora allata, the conditions of the experiment were subjected to minor variations in the hope of recruiting a positive response in a larger proportion of individuals. By increasing the number of implanted glands from one to two or three pairs, little additional effect was realized. However, the developmental response was markedly enhanced when the host animals were placed at 15 or 20° C. rather than at 25° C. after the implantation of corpora allata. It was also observed that the experimental animals which developed at the lower temperature retained a far larger proportion of pupal characters than in similar animals developing at 25° C.

3. *Inactivity of corpora cardiaca*

In the experiments just considered, the adult corpora allata were implanted together with the attached corpora cardiaca. However, in thirty-five additional preparations, the corpora allata were carefully dissected from the attached corpora cardiaca and then implanted into brainless diapausing pupae.

Table II

Tests of adult corpora allata (minus corpora cardiaca) in brainless diapausing pupae*

Adult donors	Brainless hosts	Normal development	Mixed development	No development
Cecropia	Cecropia	0	4	23
Cynthia	Cynthia	0	0	2
Cynthia	Cecropia	0	1	3
Polyphemus	Polyphemus	0	1	0
Polyphemus	Cecropia	0	0	1
Totals		0	6	29

* One to three pairs of corpora allata from male or female moths were implanted into each brainless pupa.

The results, recorded in Table II, were substantially the same as those observed in the previous experiments. Once again, a certain low percentage of brainless animals terminated diapause and transformed into pupal-adult monstrosities.

The inactivity of implanted corpora cardiaca was further confirmed in fourteen experiments in which adult corpora cardiaca were freed from corpora allata and tested, as such, in brainless diapausing pupae. No developmental response was obtained even when as many as ten pairs of adult corpora cardiaca were implanted. Indeed, in the course of twelve years of experimentation, we have never detected any trace of developmental response after the implantation of corpora cardiaca of larvae, pupae, or adults.

For present purposes it is necessary to conclude that the developmental reactions under consideration are attributable to the adult corpora allata *per se*. This implies that in a certain proportion of individuals the adult corpora allata have two effects: they first promote the initiation of adult development; they then prevent the transformation of the pupa into a normal adult moth.

4. *Effects of brain implantation*

As noted in Tables I and II, the vast majority of brainless Cecropia pupae continued to diapause when implanted with adult corpora allata. In all of these preparations the implants gave the impression of being inert. The true state of affairs is suggested by the following experiment:

Two pairs of adult corpora allata were implanted into each of five brainless Cecropia pupae. Six weeks later the pupae showed no change from their condition at the outset. Two brains of previously chilled Cecropia pupae were implanted at this time to cause the initiation of development. The latter gave rise to creatures showing large areas of pupal cuticle. In effect, the initiation of development unmasked the endocrine activity of the previously implanted corpora allata. Further information was provided by the following experiment:

Two pairs of adult Cecropia corpora allata were implanted under a facial window in each of two brainless diapausing Cecropia pupae. One month later the implants were removed and the pupae caused to develop by the injection of 125 μg. of a purified extract of prothoracic gland hormone (ecdysone).[2] Both individuals transformed into moths which retained large areas of pupal cuticle.

This experiment shows that the presence of the brain is not necessary for the secretion of juvenile hormone by adult corpora allata. In the absence of the initiation of adult development, the implants had built up a substantial titer of juvenile hormone. But the host could not signal this fact until its development was brought about by ecdysone.

5. *Experiments on isolated pupal abdomens*

Eight abdomens were isolated from diapausing Cecropia pupae. Preparations of this type remain in permanent diapause unless provided with ecdysone by injection (Williams, 1954), or by the implantation of active prothoracic glands, or by the implantation of inactive prothoracic glands plus active brains (Williams, 1952a). In the present experiment efforts were made to evoke a developmental response of isolated abdomens by the implantation of adult corpora allata—either alone, or in conjunction with brains, prothoracic glands, or injections of ecdysone.

TABLE III

Effects of implantations into isolated abdomens of diapausing cecropia

Abdomen no.	Implant	Result
1415	1 pr. adult C.C. + C.A.	No development
1447	1 pr. adult C.C. + C.A.	No development
2123	3 pr. adult C.C. + C.A.	No development
2090..	5 pr. adult C.C. + C.A.	No development
2212	$2\frac{1}{2}$ pr. adult C.C. + C.A. plus 2 chilled pupal brains	No development
1515	1 pr. adult C.C. + C.A. plus 2 pr. prothoracic glands of diapausing pupae	No development
2109	$3\frac{1}{2}$ pr. adult C.C. + C.A. plus 4 pr. prothoracic glands of diapausing pupae	Molted to form second pupal abdomen
9320	2 pr. adult C.A. (−C.C.) plus 25 μg. of crystalline ecdysone	Molted to form second pupal abdomen

Table III summarizes the several types of preparations. It is of particular interest and importance to note that no development took place when the abdomens received only adult corpora allata. We have checked this finding in twelve additional experiments performed on isolated Cynthia abdomens; in this case the pupal abdomens were distributed at 15, 20, and 25° C. after the implantation of two to five pairs of corpora allata derived from adult Cecropia or Polyphemus. In short, no trace of development was ever observed in response to the implantation of adult corpora allata *per se*. The same negative result was also recorded in an experiment where adult corpora allata were implanted along with active brains.

The preparation numbered 9320 in Table III is of particular interest. Here, two pairs of adult corpora allata were implanted into an isolated abdomen. A month later 25 μg. of crystalline ecdysone were injected. Development began within two days. Within the following ten days the pupal abdomen transformed and molted into a second pupal abdomen (see Figure 5). This result was duplicated in two additional experiments utilizing Cynthia abdomens. It is clear that ecdysone is the prime-mover in the developmental response and that the juvenile hormone is inactive in the absence of ecdysone.

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[2] I am indebted to Dr. Peter Karlson for supplying highly purified preparations of ecdysone.

Attention is now directed to preparation 2109 in Table III. This pupal abdomen received implants of adult corpora allata plus diapausing pupal prothoracic glands. Precisely the same result was observed as after the injection of ecdysone: the pupal abdomen molted and transformed into a second pupal abdomen. In this case it seems necessary to conclude that the corpora allata activated the diapausing prothoracic glands—that, in this sense, a hormone from the corpora allata had substituted for the brain hormone. However, there is no indication in Table III that this corpus allatum hormone can substitute for ecdysone itself.

6. *Tests of adult corpora allata in previously chilled pupae*

The results considered to this point lead to the prediction that adult corpora allata should be uniformly active when tested in previously chilled pupae just prior to the initiation of adult development.

During the past ten years this prediction has been confirmed on a large scale. The experimental series includes ninety-eight preparations in which corpora allata of male and female moths of Cecropia, Polyphemus, and Cynthia were tested in chilled pupae of each of the same three species. All except eight animals gave rise to adults retaining pupal characters. In the eight negative tests the implanted glands had been derived from elderly adults just prior to death.

There was a rough correlation between the number of implanted glands and the degree to which pupal characters were preserved—a finding which will be considered in further detail in the following paper. Moreover, as was true in the earlier experiments on brainless pupae, the effects of the implanted corpora allata were amplified when the host pupae were placed at 15 or 20° C., rather than at 25° C., immediately after the implantation.

The retention of pupal characters was extreme in many of the test animals. As shown in Figure 2, the pupa transformed into a second pupa which showed only traces of adult characteristics. In several experiments performed on Polyphemus and Cecropia, the secondary pupa molted into a tertiary form. In this case, the pupal characteristics were less prominent after the second molt than after the first.

None of these animals was viable for any prolonged period after transforming into mixed forms. Although the old pupal cuticle became thin and crisp and the ecdysial lines were eroded to the surface, spontaneous escape from the old pupal cuticle occurred only in individuals showing minimal retention of pupal characteristics. All other animals remained enveloped in the old pupal cuticle until they died or were sacrificed.

In many of the individuals the molting process proceeded to a normal terminal phase accompanied by a complete breakdown of the old endocuticle and a partial or complete resorption of the molting fluid. Yet, for some unexplained reason, the insect failed to undertake the vigorous muscular efforts that accompany a normal ecdysis. It did not "try to molt" even though it possessed the nervous and muscular equipment to do so. The use of forceps was therefore necessary to peel off the old pupal exuviae.

In many individuals it was difficult or impossible to withdraw the lining of the old tracheal tubes through the spiracular openings. Indeed, in the case of Cecropia, the larger branches of this old system became stiff and melanized and therefore incapable of being shed. The net effect is that the juvenile hormone is a lethal agent for all these Saturniid pupae.

7. *Inactivity of killed corpora allata*

The high activity recorded for implanted adult corpora allata suggested the possibility that substantial amounts of hormone might be stored within the glands themselves. This prospect was tested in five experiments. In one experiment eight adult Cecropia corpora allata were frozen and thawed twice at −40° C. and then implanted into a previously chilled pupa. Normal development ensued.

In four other experiments adult corpora allata, in numbers ranging from 9 to 44, were homogenized in 0.1 ml. of insect Ringer and then introduced into four previously chilled pupae. All four animals developed into normal adult moths. Evidently, little or no hormone is stored in the living gland, for the activity of a single living adult corpus allatum was not duplicated by the implantation of up to forty-four dead glands.

Discussion

1. *Secretion of the juvenile hormone by the adult corpora allata*

The experimental results demonstrate the endocrine activity of the corpora allata of Cecropia, Polyphemus, and Cynthia moths. As is amply evident in Wigglesworth's (1954) recent review, this finding is consistent with the picture presented in all other insects that have been studied in detail including several families of Lepidoptera. In the Cecropia silkworm the corpora allata, when removed and tested, are found to be more active in the adult moth than at any other stage in the life history (Williams, unpublished data). Moreover, there is general agreement that at least one of the secretory products of the adult corpora allata is the same juvenile hormone which is secreted weeks or months earlier by the corpora allata of the immature insect. This conclusion was first proposed by Pflugfelder (1938a, 1938b) and Pfeiffer (1945), and will be further documented in the subsequent papers in this series.

2. *The role of the juvenile hormone in adult moths*

We have been unable to detect any function for the corpora allata in the pupal or adult stages of these Lepidoptera. Thus, as we have seen, the corpora allata can be removed from pupae of either sex without disturbing the development of normal, viable, sexually mature moths. These findings are the same as those reported for *Bombyx mori* by Bounhiol (1938) and Fukuda (1944). The present study enlarges the negative evidence by showing that the absence of corpora allata fails to interfere with the maturation of functional gametes and the production of normal offspring.

The situation in the Lepidoptera therefore departs from that described for most other orders of insects where the corpora allata are necessary for the deposition of yolk in the adult female and for the secretory activity of the accessory glands in the adult male (for summary, see Wigglesworth, 1954, pages 77–80). In the Lepidoptera which have been studied, all these functions can go forward in the absence of corpora allata. For the sexual maturation of both males and females all that is required is the presence of prothoracic gland hormone (ecdysone). The brain hormone is also unnecessary for the sexual maturation of these silkworms. Pupae from which the brain, corpora cardiaca, and corpora allata have been removed develop into sexually mature moths after the injection of crystalline ecdysone (Williams, 1954).

Adult Lepidoptera therefore present the paradoxical picture of the presence of highly active corpora allata for which there appears to be no apparent function. However, it is worth recalling that corpora allata have been tested only in species of adult Lepidoptera which are short-lived and unable to feed. In adults of the giant silkworms, as in the commercial silkworm, functional mouth-parts are absent. Consequently, the duration of the adult stage is greatly curtailed: ripe eggs must be ready for oviposition at the time of adult emergence. In short, the absence of mouth-parts has enforced on these short-lived moths a precocious maturation of the gonads during the course of pupal-adult development. Indeed, months before the development of the adult moth, the proteins which later appear in the yolk of the eggs are already present in high concentrations in the blood of the diapausing pupa (Telfer, 1954).

It is among the feeding, long-lived species of adult Lepidoptera that one would anticipate a gonadotropic function for the corpora allata akin to that seen in most other orders of insects. This inference is in accord with the histological studies of Kaiser (1949) on long-lived butterflies of the genus *Vanessa*. Presumably, in the Ephemeroptera and other non-feeding adults one should find the same picture as presented by the Saturniidae.

The absence of functional adult mouth-parts is clearly a secondary affair in the evolution of the Lepidoptera. Indeed, the very same moths contain digestive tracts of normal organization, but of no apparent function. Evidently, the presence of active corpora allata is a memento of a more primitive endocrinological situation.

3. *Biological role of the juvenile hormone*

The juvenile hormone plays no role in the transformation of a pupa into an adult moth. All that is required is that the juvenile hormone be absent throughout the early phases of this transformation (Williams, 1952b). This conclusion is in line with the finding that the corpora allata are inactive throughout the entire pupal stage and during the first two-thirds of adult development (Williams, unpublished data).

A pupa can be supplied with juvenile hormone by the implantation of living, active corpora allata obtained from larvae or adults. However, as demonstrated in the experiments on isolated pupal abdomens (Table III), the juvenile hormone has no effects in the absence of the prothoracic gland hormone, ecdysone. Only when the abdomen is provided with this hormone can one detect any action of the implanted corpora allata. The outcome is that the pupal abdomen terminates diapause, molts, and transforms into a second pupal abdomen (Fig. 5).

Substantially the same result is seen in experiments performed on brainless diapausing pupae. Here again the implantation of adult corpora allata is inconsequential unless ecdysone is supplied by injection or by the secretory activity of the animal's own prothoracic glands. The juvenile hormone then opposes the transformation of the pupa into an adult moth. The result (Figs. 3 and 4) is a creature showing to varying degrees a retention of pupal characters of the type previously described by Piepho (1952) and Williams (1952b). When the titer of juvenile hormone is high, then one may witness the formation of a *bona fide* second pupal instar—a phenomenon hitherto unknown in any insect (Fig. 2). But, even in the presence of the highest concentrations of juvenile hormone, we have never observed in this material the reappearance of larval characters such as described in *Rhodnius* (Wigglesworth, 1954, 1957, 1958).

4. *Mimicking of brain hormone*

In a certain proportion of brainless diapausing pupae the implantation of active corpora allata causes the termination of diapause and the initiation of adult development. This result is not seen in isolated pupal abdomens or other preparations lacking prothoracic glands. But, as noted in Table III, the developmental reaction becomes possible if an isolated abdomen receives active corpora allata plus inactive prothoracic glands, or active corpora allata plus an injection of ecdysone (Fig. 5). Moreover, in numerous experiments to be described on a later occasion, the development of brainless diapausing pupae has been provoked by the injection of crude or purified extracts of juvenile hormone. Evidently, under certain undefined conditions, a hormonal secretion of the corpora allata can activate the prothoracic glands and, in this sense, mimic the function of the brain hormone. Whether this hormone is the juvenile hormone or some further secretory product of the corpora allata is impossible to state at the present time. A decision on this point will become possible only when the juvenile hormone is isolated and tested in pure form.

The finding that the corpora allata can turn on the prothoracic glands has an obvious bearing on the endocrine control of larval molting. If the corpora allata can activate the pupal prothoracic glands, there is no reason to suppose that they cannot do so in the immature larva.

We begin to see a multiplicity of agencies which can promote the secretion of ecdysone by the prothoracic glands. The brain can turn on the prothoracic glands. Ecdysone can turn on the prothoracic glands (Williams, 1952a, 1954). And, evidently, under certain undefined conditions, so also can the corpora allata. Nature has apparently found it prudent to surround the prothoracic glands by a net-work of controls. The present study suggests that the corpora allata are a part of that net-work.

SUMMARY

1. Juvenile hormone is secreted in high concentration by the corpora allata of the adult Cecropia moth.

2. Notwithstanding this fact, the juvenile hormone has no apparent function in the adult moth. Extirpation of the corpora allata in the pupal stage fails to interfere with the production of normal moths whose gametes give rise to normal offspring.

3. The corpora allata are inactive during the entire pupal stage as well as during the first two-thirds of adult development. If active corpora allata are implanted into a pupa just prior to the initiation of adult development, the juvenile hormone acts to oppose the differentiation of the adult moth. Development gives rise to an insect showing a mixture of pupal and adult characters. In the presence of high concentrations of juvenile hormone the pupa molts and transforms into a second pupa showing only traces of adult characters.

4. The biological action of juvenile hormone is seen only in the presence of active prothoracic glands or their secretory product, ecdysone. Isolated pupal abdomens fail to respond to juvenile hormone unless ecdysone is simultaneously present. When both hormones are present, the pupal abdomen terminates diapause, molts, and transforms into a second pupal abdomen.

5. Evidence is presented that the corpora allata secrete a factor which can mimic the brain hormone and activate the prothoracic glands. This finding is considered in relation to the endocrine control of larval molting.

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EMBRYOLOGICAL DEVELOPMENT OF THE POLYCHAETOUS ANNELID, DIOPATRA CUPREA (BOSC)[1]

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Insofar as the writer knows, the normal embryology of *Diopatra cuprea* has never been completely worked out. The main trouble seems to have been that investigators, with the exception of Just (1922), have found that it is difficult to activate the eggs of this species even when they appear ripe. Andrews had similar difficulty with the eggs of the closely related species, *Diopatra magna* (since designated *Onuphis magna*). He made the statement (1891b, page 115) that "attempts at artificial fertilization were unsuccessful" although the eggs seemed ripe as indicated by their size and the large numbers present packing the coelom, as well as the occasional finding of similar eggs amongst the larvae in the egg masses which he found during the breeding season. However, Just (1922), in a paper concerned primarily with raising mature *Platynereis megalops* from eggs, noted (page 477), "Though it is usually stated that artificial insemination of Diopatra eggs is not possible, every attempt made by the writer . . . was successful," and that he reared *Diopatra cuprea* to a length of 4 cm. No record of development was given.

The problem of activation has remained a significant one throughout the course of this investigation. With perseverance (particularly initially) larvae from many batches of eggs have been raised during the course of several summers to a stage where 6 sets of setae have been formed and, in the summer of 1958, a few were raised to a stage with 7 sets of setae. Thus far two abstracts have been published on this work (Allen, 1951, 1953) and more recently Costello *et al.* (1957) have included some additional previously unpublished data (furnished by the present writer) in their book on handling marine eggs and embryos.

The study of the development of *D. cuprea* is still incomplete but enough additional material has recently been worked out so that it was thought advisable to publish a more detailed account of development than has thus far been done. There is little material in the literature on the development of the genus, Diopatra. As further observations on living material were made, the confusion in the literature surrounding the development of the species, *D. cuprea*, became more apparent.

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Observations made during the present study suggest that most, if not all, of the material which has been published on the development of *Diopatra cuprea* has been incorrectly attributed to this species, so that the investigations of the writer may represent the only material published on the development of this polychaete.

MATERIAL AND METHODS

The adult worm. The characteristics and habits of the adult worms of this species have been described by various investigators (Andrews, 1891a; Sumner, Osborn and Cole, 1911; Hartman, 1945, 1951; *et al.*). The parchment-like tubes of these polychaetes, which are found in the intertidal zone, go down two to three feet into the substratum. When disturbed the worm retreats into the tube so that in digging for the adults one rarely obtains the whole worm. As a result, the posterior tip with its four anal cirri is rarely seen. The head bears five occipital tentacles and two shorter frontal tentacles. Larvae have been raised to a stage when the five occipital tentacles and two anal cirri are noticeable. Males which are sexually mature are cream to yellowish in color as a result of the sperm packed in the coelom. In males with fewer sperm only the parapodia are yellowish in color. Females when sexually mature are usually grey-green due to the color of the eggs (which have a green pigment) packed in the coelom. This species is plentiful in Woods Hole waters. Most of the collecting for this investigation was done at Northwest Gutter, Hadley Harbor, Massachusetts, and some of it was done in the harbor at North Falmouth and at Woods Hole, on the Buzzards Bay side. The adults for the most part were kept in aquaria in running sea water. The worms were fed every day or two with pieces of the mussel, Mytilus.

Procuring and handling living developmental stages. The writer has raised larvae of *Diopatra cuprea* from mid-June through August following artificial fertilization. The problem of activation of apparently ripe eggs was present throughout this period but artificial insemination was more successful in June and July than in August. This is contrary to the remark of Bumpus (1898, page 855) that "the ova are nearly ripe in August."

During the breeding period of a sexually mature worm, the coelom becomes packed with gametes. When eggs or sperm are needed, the posterior end of a worm is exposed by cutting the end of the tube with scissors. The exposed portion is then held lightly with forceps. This usually results in the worm's pinching off its posterior segments. Eggs were obtained from the isolated posterior sections by slitting the body wall along the bases of the parapodia with No. 5 watchmaker's forceps. Eggs thus obtained were washed in Syracuse dishes with sand-filtered sea water. In general, spermatozoa were obtained by making a small slit at the base of a parapodium with a No. 5 watchmaker's forceps and diluting the "dry" sperm with sand-filtered sea water. Under the dissecting microscope ripe sperm were observed to be active immediately. Polyspermy should be avoided.

Within a few minutes after insemination the eggs were washed several times with sand-filtered sea water. Usually they were given fresh sea water one to two hours later. If development were normal, ciliated larvae developed at room temperature within three hours after insemination. At this stage larvae usually were transferred to stender dishes and placed on the sea water table in a moist chamber with 90% sea water in the bottom. The water was changed at least once a day thereafter.

Apparently egg laying in *D. cuprea* is a phenomenon rarely observed (Sumner *et al.*, 1911). In only one instance did the writer observe natural egg laying in the laboratory. This was on the evening of June 23, 1949. A worm tube was picked up and eggs were immediately released in a transparent, only slightly viscous, jelly which dissolved readily in sea water. The eggs were fertilized artificially and almost 100% cleaved. Only a few other times in the experience of the writer has fertilization approached 100%, as the method of artificial insemination described is frequently unsuccessful. To get a batch of eggs with 50% of the eggs cleaving is good.

Observations were made on living stages with the dissecting and compound microscopes and, in the summer of 1958, additional observations were made with the phase microscope. For study and for photomicrographs the ciliated stages were slowed down with a little dry MS-222 (tricain) added with a dissecting needle to a drop of filtered sea water containing the larvae (optimal concentrations for quieting various larval stages were not determined).

For the setal studies the larvae were placed on a slide in a drop of filtered sea water and then a cover slip was applied. They were examined briefly under a magnification of × 430 and then left to dry a little. This treatment in many cases spread out the setae which were then studied in more detail under × 430.

Handling of fixed material. Various stages were fixed, paraffin-embedded, and serially sectioned (usually at 7 or 10 micra). Whole mounts of stained and unstained stages were also made. The fixatives used for the early stages were usually Allen's B-15 or Bouin's, and for later larval stages Schaudinn's or Bouin's heated to 60° C. A series was also fixed in Meves'. A variety of stains was tried including Heidenhain's hematoxylin, Harris' hematoxylin, acetocarmine, alum-cochineal, Giemsa's, toluidin blue, and Feulgen's. Sections and whole mounts usually were mounted in Permount or Canada balsam. It was considered important to use such whole mounts to make a cell lineage study through at least the early cleavage stages. However, the method which had given excellent results with cleavage stages of the gastropod, Crepidula, failed completely with Diopatra. Various other techniques have been tried, including pre-treatment to remove lipids or ribonucleic acid, either of which might take up the stain in the cytoplasm. To date a technique has not been developed that would stain the chromosomes and enable one to follow the orientation of the spindles without staining the cytoplasm.

Normal Development

The writer has indicated already (1951) that the cleavage of *Diopatra cuprea* occurs with amazing rapidity, functional cilia being formed within three hours after insemination. Prior to this age, it is difficult to construct a time table of development because there is considerable variability among different batches of eggs and also among different eggs in the same batch, particularly in cases in which low percentages of fertilization occur. The following represents a slight elaboration of the schedule recorded in Costello *et al.* (1957) which is based on the writer's data obtained over several summers. The times are recorded from insemination at temperatures of 21–24° C.

Stage	Time
First polar body	15–20 minutes
Second polar body	20–30 minutes
Two- to four-cells	40–60 minutes
Eight-cells	50–90 minutes
Mid- to late cleavage	90–120 minutes
Functional cilia	3 hours
Apical tuft (apparent in some)	8–9 hours
Apical tuft (present in all normal larvae)	12 hours
Rotating trochophores	24 hours
2 to 3 sets of internal setae	36 hours
3 sets of external setae, no tentacles	2 days
4 sets of external setae, some with 3 tentacles	3–4 days
5 sets of external setae, 5 tentacles	4½–5½ days
6 sets of external setae, 5 tentacles	6½–8 days
7 sets of external setae, 5 tentacles	13–17 days (typical?)

The various stages of normal development are described in more detail below.

The unfertilized egg. In *Diopatra cuprea* the unfertilized egg is oval. After its growth period the average size of the egg is approximately 235–240 × 205–210 micra (Fig. 2). (Andrews, 1891b, gives the diameter of this egg as 400 micra; however, the above dimensions are based on repeated measurements by the writer.) In living eggs the germinal vesicle is visible as a lighter region near the animal pole. Surrounding it is an area of non-yolky cytoplasm in which are suspended bright green pigment granules. External to these are yolk granules which increase in number towards the vegetal pole. Their accumulation thus establishes a visible animal-vegetal gradient and makes the egg very opaque. In reflected light under the dissecting microscope the eggs *en masse* in some batches are creamy yellow or creamy white; in other batches, eggs have a greenish hue. With dark-field (under low power) the eggs are a rich yellowish cream color with brilliant green granules obvious around the germinal vesicle. The differences in color apparent under the dissecting microscope are due to the relative amounts of green pigment and yolk. The egg has a clearly defined membrane, approximately 3 micra thick, which appears to be perforated by radial pores when viewed under the compound microscope.

A curious feature of the development of these eggs is the two strings of cells attached to them during their growth period in the coelom. Andrews (1891b) described these follicle cells and states (page 113) that "these objects were at first mistaken for parasitic algae." These "nurse" cells are transparent (Fig. 1) and bear a striking resemblance to blue-green algae. However, each algal-like cell has a relatively large nucleus with a prominent nucleolus (Fig. 16). In the very small oocytes bearing these "nurse" or follicle cells, the pigment is a brilliant green as there is little or no yolk to mask it. These smaller eggs have a central nucleus (Fig. 16). Subsequently, with the differential accumulation of yolk, the nucleus becomes excentrically located, coming to lie near the animal pole (region indicated in Figure 17).

Apparently the follicle cells are not lost until the end of the growth period in oogenesis as a few full-size eggs have been observed with these algal-like strings attached. Andrews (1891b) has observed that these cell strings are retained in *D. magna* until near the end of the growth period.

Fertilization. The egg is fertilizable at the germinal vesicle stage. The first indication of fertilization is the lifting off of the egg membrane to form the fertilization membrane. The perivitelline space is slight, being most obvious in the region of the animal pole. The germinal vesicle becomes less and less distinct as the perivitelline space forms. Usually the first polar body is given off within 20 minutes after insemination and the second within 30 minutes after insemination. A small pigment-free area around the animal pole marks the position where the second polar body will pinch off (Fig. 2). The polar bodies are small (Fig. 4), the second polar body being somewhat larger than the first. Figure 17 shows a section of an egg in metaphase I (the polar bodies are not visible).

Cleavage. Occasionally the two-cell stage may be observed 30 minutes after insemination but usually the first cleavage is not completed until 40 minutes or so after insemination. The first cleavage furrow is meridional, cutting through the animal pole first (Fig. 3) resulting in two blastomeres of unequal size, the AB being somewhat smaller than the CD blastomere (Figs. 4 and 18). There is some variation in the size difference between the first two blastomeres. Blastomeres AB and CD often divide about the same time as seen in living stages and sections. In some cases the larger blastomere appears to divide first, as three-cell stages may be observed (Fig. 19). It is possible, however, that these three-cell stages represent abnormal development. The four-cell stage shows a cross furrow with the arrangement of cells typical of spiral cleavage (Fig. 5). In living stages the nuclei appear as lighter regions. The third cleavage results in an eight-cell stage with four somewhat smaller micromeres being polar in position (Fig. 6). Cleavages beyond these first few are amazingly rapid. A mid-cleavage stage is shown in Figure 7. During late cleavage the blastomeres are held firmly together within the original egg membrane, and a vacuolated peripheral area is appearing (Fig. 20).

Early ciliated stages (3 to 12 hours). These stages are approximately the size of the unfertilized egg. While there is no increase in mass, cells are continuing to divide. Gastrulation in these very rapidly developing early stages may be occurring by the time cilia have differentiated and probably takes place primarily by epiboly.

Functional cilia penetrate the egg membrane within three hours after insemination. They appear to push through the pores noted above in the membrane of the unfertilized egg. It is difficult to be certain of the ciliary distribution even when using the phase contrast microscope, but cilia appear to cover the entire surface except for two areas, a disc at the posterior end and the region around the future apical tuft. The cilia thus appear to form a very broad band involving most of the larva. At this stage peripheral vacuolated cells form four anterior plates which surround, and appear distinct from, a central mound of denser cells (Figs. 8, 9, 21, 23). A few small pigment spots may be observed in living larvae. Normal larvae move in place for awhile but very shortly become surface swimmers. They swim forward, at the same time spinning clockwise on the longitudinal axes when viewed from the animal pole.

By slowing down swimmers with MS-222 and observing them with the phase microscope (using dark-field which gives a strikingly beautiful picture), it is possible to get a "head-on" view of the former animal pole region. If the larvae spin slowly enough, one can see what looks like a diagrammatic representation of both the apical rosette (blastomeres $a_{1.3}$–$d_{1.3}$) and annelid cross (compare Plate XI, Figure 18, on Amphitrite in Mead's paper, 1897, page 311, or Figure 196-5, based on Nereis, in Borradaile and Potts, page 283). Blastomeres $a^{1.2}$–$d^{1.2}$ are less dis-

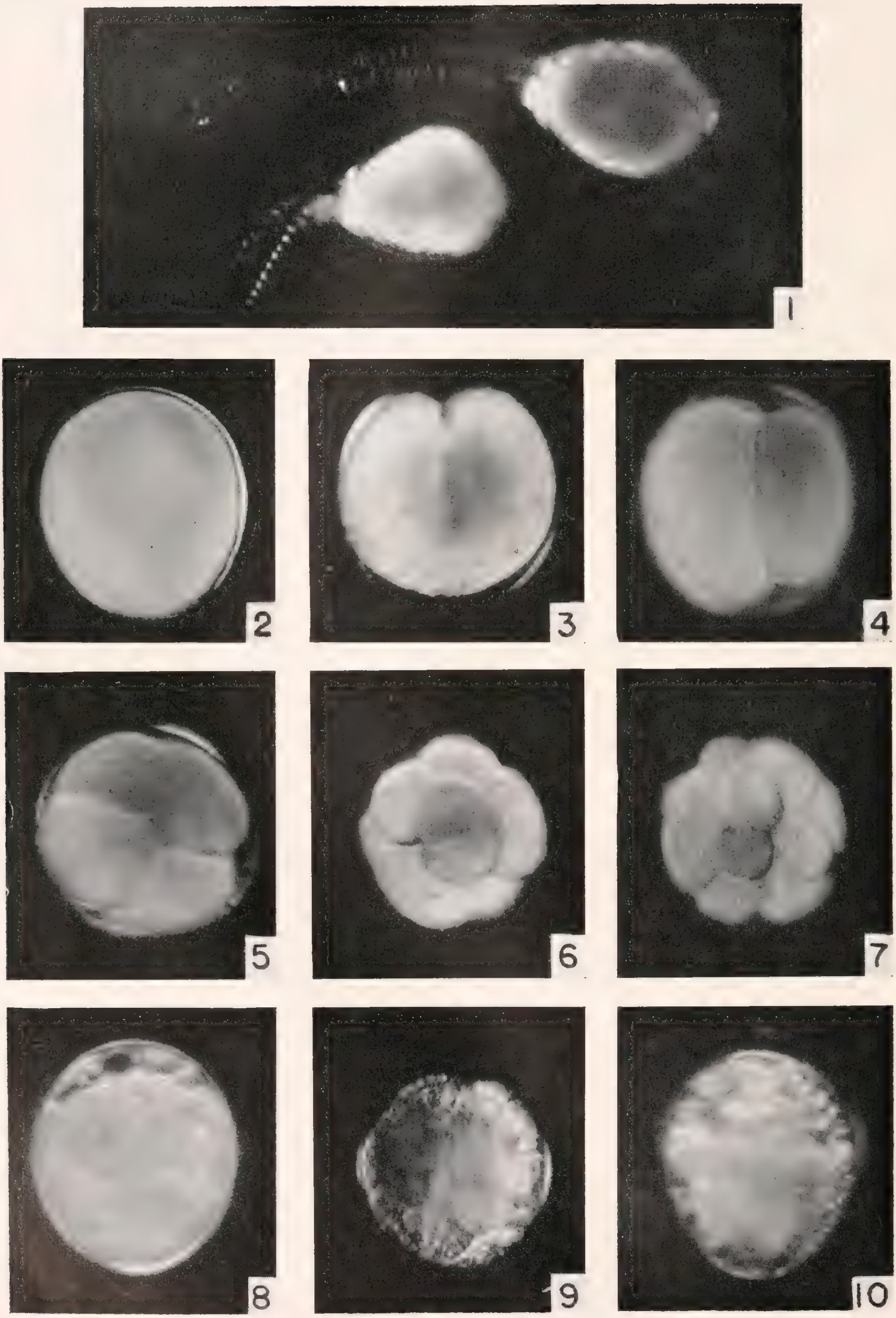
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PLATE I

tinct but can be made out (blastomeres $a^{1.2}$–$d^{1.2}$ and $a_{1.3}$–$d_{1\ 3}$ are apparently designated as a^{12}–d^{12} and a^{13}–d^{13} in Borradaile and Potts). The four anterior plates of cells appear to arise from the four groups of prototroch cells and thus mark the position of the prototroch proper beneath them. The apical rosette forms the tip of the central mound of cells. Sometimes one or two globules (probably polar bodies which have not yet disintegrated) are seen in the space between the central mound and the membrane (Fig. 21).

The central mound in some 7-hour swimmers has grown almost to the animal region of the membrane. The apical tuft, in some larvae at least, appears one to two hours later. The cilia of the apical tuft have their origin from the central cells at the tip of the mound (in the few cases measured, cilia were approximately 40 micra when first formed). Their origin is not surprising, for, as noted above, the central cells make up the apical rosette which has been shown in other polychaetes to become the apical organ of the trochophore.

Continuous with the four anterior vacuolated plates, but extending posteriorly, are at least four yolk plates. Their formation leaves a space between them and the medial endodermal yolk mass of the larva. Anterior vacuolated plates and posterior yolk plates merge in the peripheral portion of the larval mass at about the equatorial level. Yolk spheres similar to those in these curious thin plates can be traced in serial sections from the posterior part of the larval mass peripherally and anteriorly (where some are observed at the base of the vacuolated plates) and then posteriorly just under the cuticle where they form thin plates. The yolk plates thus appear to arise from the posterior part of the larval mass (original vegetal hemisphere). The narrow spaces between each plate and the median mass are continuous with each other posteriorly and are visible as slits in some 12-hour larvae (and older) when they rotate. All normal larvae of 12 hours have a prominent apical tuft (one measured approximately 100 micra) which can be seen under the dissecting microscope. Larvae swim rapidly about the antero-posterior axis much as before with the apical tuft directed forward.

All figures are photomicrographs taken with a Makam camera. Figures 1 through 15 are all of living stages, taken at × 100, without a cover glass except for Figure 15. Moving stages were quieted with MS–222 (tricain). Figures 16 through 28 are all of sectioned material, taken at × 352 except for 27 and 28 which were taken at × 220. Figures 29 through 34 are all photomicrographs taken from "dry" mounts of larvae at × 430.

PLATE I

EXPLANATION OF FIGURES

FIGURE 1. Developing eggs before their growth period is completed, showing algal-like strings of cells. FIGURE 2. Unfertilized egg showing lighter granular area at the animal pole where the polar bodies will pinch off. FIGURE 3. Fertilized egg with cleavage furrow beginning first at the animal pole. FIGURE 4. Two-cell stage, showing that the CD blastomere is somewhat larger than the AB. Note the fertilization membrane and one of the polar bodies. FIGURE 5. Four-cell stage viewed from animal pole, showing the cross furrow characteristic of spiral cleavage. FIGURE 6. Eight-cell stage in two tiers, four slightly smaller micromeres towards the animal pole. FIGURE 7. Early to mid-cleavage showing individual blastomeres. FIGURE 8. Early swimming stage, approximately four hours old, showing two of the four plates surrounding the central mound. FIGURE 9. "Head-on" view of stage similar to Figure 8, showing four plates of cells (one at lower right clear) surrounding the mound. FIGURE 10. Trochophore, approximately 28 hours old, with apical tuft and prototroch (haze at right represents the beating cilia).

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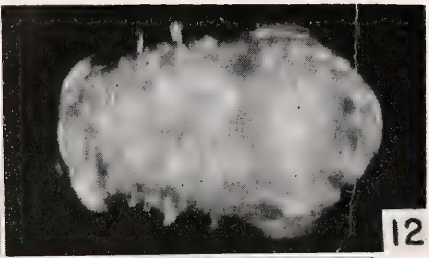
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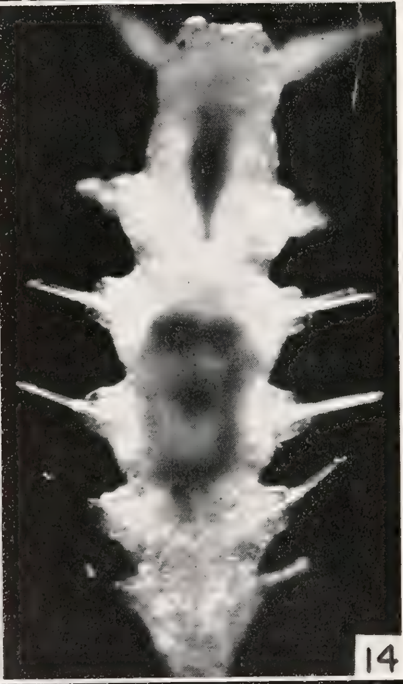
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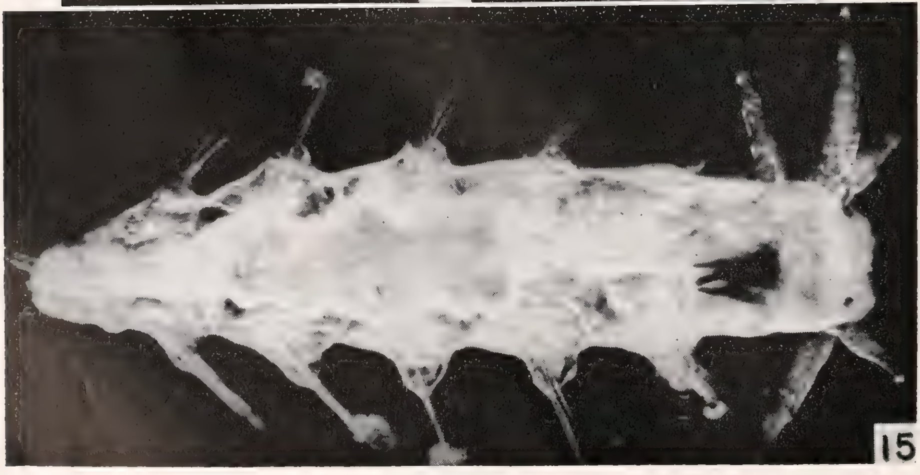
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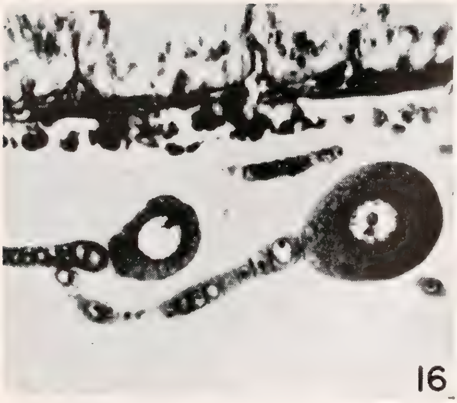
Plate II

Trochophore stage, 24 hours old. Larvae of this stage are still approximately the size of the unfertilized egg and are positively phototactic active swimmers, rotating clockwise as in the preceding stages. Anteriorly, they have two red eyespots and a prominent apical tuft, 60–70 micra or more in length, consisting of several long cilia surrounded by a ring of shorter cilia. The body is still covered with cilia except for the small disc at the posterior end and a small area around the apical tuft (Fig. 10). The cilia appear somewhat longer in the region of the developing prototroch and telotroch. The denser central mass of cells represents the differentiating yolk-laden mid-gut (Fig. 22). In living stages a slight indentation observed on one side may represent the stomadeum. A few dark pigment spots (green in dark-field) in the region of the broad prototroch tend to mask the pharynx in living larvae. In some larvae the slit-like spaces formed between the posterior yolk plates and the underlying larval mass are still obvious; in others, growing cells have obliterated the slits so that the peripheral yolk plates are caught between the mesodermal bands and the cuticle. The yolk plates are then visible as a line of yolk spheres just beneath the cuticle (barely visible in Fig. 22). Thus, posteriorly, the layers from inside out are the central yolk mass, mesodermal bands, slits (in some instances), yolk plates, and larval membrane (Fig. 22).

Post-trochophore stage, 36 hours old. This stage is usually little longer, though somewhat narrower, than the preceding and is characterized internally by the beginnings of two to three sets of setae and the formation of glandular cells (probably mucous in nature; Figs. 24, 25, 26). The larvae are strongly positively phototactic as evidenced by their swarming toward the light. They have prominent red eyespots and a well developed apical tuft (most of the cilia are approximately 85 micra, the longest measured being approximately 100 micra). The cytoplasm at the level of the broad prototroch has a bubbly appearance due to refractile droplets which tend to obscure the pharynx. Other external features are the narrow telotroch, short cilia between the proto- and telotrochs, and possibly a posterior tuft of cilia (a suggestion of this last was observed only twice, with the phase microscope). Yellowish pigment may be observed scattered over the surface. Visible under the cuticle posteriorly are the peripheral yolk plates. The gut from an external view is similar to that in the preceding stage, forming a darker central mass (yellow in reflected light). A posterior indentation may represent the proctodeum, as the hind-gut has not yet formed. The larvae appear to be flattened slightly on the ventral surface. Serial sections reveal the pharynx, differentiating setae, mucous cells, and four large posterior vacuoles which probably represent the free ends of mucus-secreting cells (Figs. 24 and 26).

PLATE II
EXPLANATION OF FIGURES

FIGURE 11. Larva of 3½ days with four sets of setae (the fourth set has retracted). Visible are two eyespots at right, two posterior vacuolated cells at left, and darker mid-gut region between the setae. FIGURE 12. Swimming larva, also 3½ days old, with the fourth set of setae just emerging. Note the beating prototroch at eye level and the telotroch at left. FIGURE 13. Larva of approximately four days, showing four sets of setae, three dorsal tentacles beginning to form, and black jaws visible through the body wall. FIGURE 14. Larva of six days with five sets of setae and dorsal tentacles elongating. Note the eyespots, dark jaws, and dark mid-gut region. FIGURE 15. Larva of seven days, with five sets of setae, photographed with cover glass. Visible are two eyes, "knobby" tentacles, black jaws, light mid-gut region, and two anal cirri.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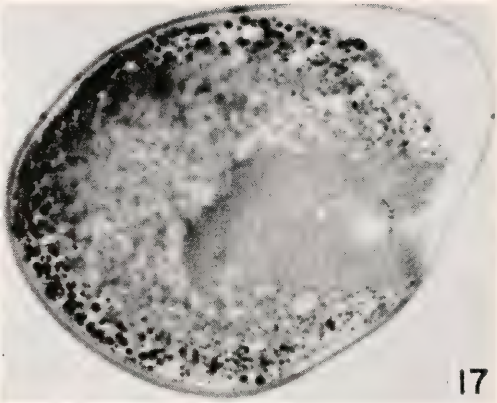
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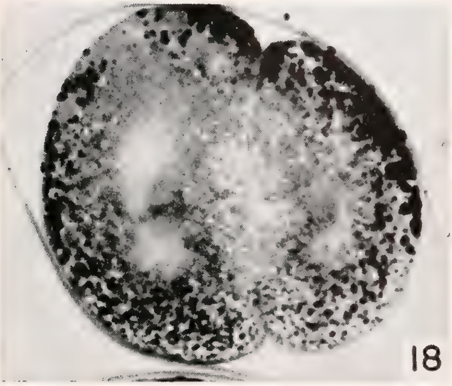
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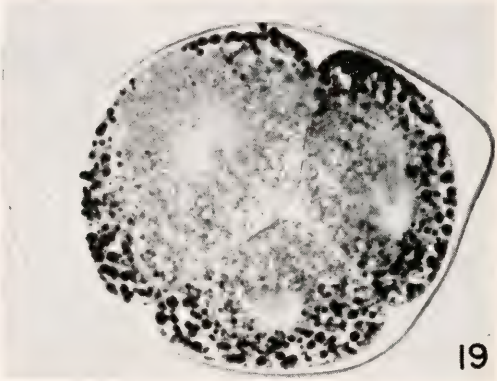
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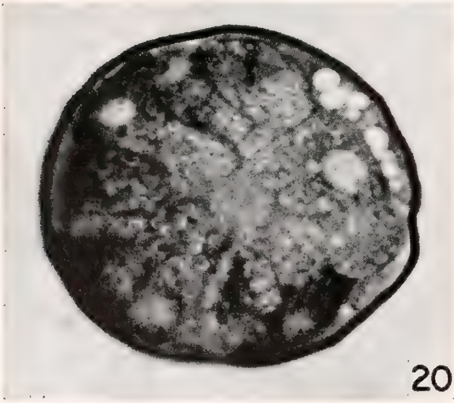
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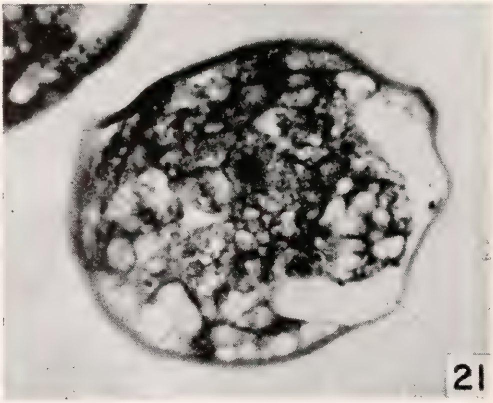
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PLATE III
EXPLANATION OF FIGURES

FIGURE 16. Section of two young eggs in the coelom, showing attached algal-like strings of cells (second string not in plane of section), the nucleus and prominent nucleolus in the egg and in each of the "nurse" cells. FIGURE 17. Fertilized egg in metaphase I, showing the

Larvae of 2 days, 8–12 hours. These larvae, about the same width as the preceding, have elongated by about 100 micra and measure approximately 325 × 200 micra (in measurements of larvae, widths indicate the broadest portion). The tendency of some larvae to settle on the bottom at this stage seems to be correlated with the secretion of mucus; other larvae, however, are still actively rotating, positively phototactic swimmers. Their invariable swarming towards the light makes changing the water easy at this stage. The larvae usually have differentiated three sets of setae externally (sometimes only two), with a fourth set forming internally in some. The third set, though extending externally, may be incompletely formed (see Table I).

Larvae have two prominent red eyespots and several pigment spots anteriorly. The apical tuft, though reduced, is still prominent, being roughly 55 micra long. The anterior arms of the opaque Y-shaped mid-gut surround the colorless pharynx. Scattered black pigment spots can be seen in surface view. The prototroch is still present as is the telotroch of longer cilia, and between them are shorter cilia. Rarely seen, but very clear when observed with the phase microscope, is a little patch of cilia just posterior to each set of setae. The characteristic refractile droplets are still present at the widest part of the prototroch and this area appears continuous with the mid-gut region. The hind-gut is not clearly defined.

Larvae of 3 days, 8 to 12 hours. Larvae of this stage are slightly longer and usually somewhat narrower than those of the preceding stage (for example, one measured 400 × 180 micra). A few are still swimming and are positively phototactic, but most tend to crawl on the bottom, secreting mucus as they do so. They sometimes stick together in clumps in which case they should be separated before they die. Some have formed transparent slime tubes. Usually four functional sets of setae are visible externally (Figs. 11 and 12) and the parapodium of the first setigerous segment has two protrusions, a finger-like postsetal lobe and a shorter presetal lobe (Fig. 33). A tuft of cilia, rarely observed, is present at the base of each parapodium. An apical tuft is still prominent but is often missed even with the phase microscope, for it tends to bend backward when slowed with MS-222. The fairly broad prototroch extends from the anterior level of the eyespots to just anterior to the first set of setae (compare Figures 11 and 12). The prominent telotroch lies just posterior to the last set of setae (Fig. 12). Incipient jaws have differentiated which have an extra toothed plate on one side of the otherwise symmetrical maxillae (similar to Fig. 29). This asymmetry of the jaws is characteristic of the adult. These larval jaws are movable indicating that pharyngeal muscle is differentiating. Peripheral vacuolated mucous cells are clearly defined. Two of the large posterior vacuoles may be visible externally (Fig. 11). The broad anterior region, with its bubbly cytoplasm, still appears

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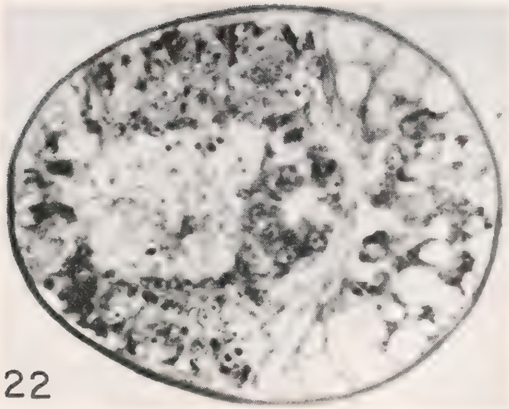
contrast between yolky and non-yolky cytoplasm. FIGURE 18. Two-cell stage in metaphase of second cleavage showing that the CD blastomere is larger than the AB. Note the fertilization membrane. FIGURE 19. Three-cell stage showing that the CD blastomere sometimes cleaves before the AB. This may represent abnormal development. FIGURE 20. Blastomeres of late cleavage held firmly within the egg membrane. The peripheral vacuolated region is beginning to appear and one blastomere is in metaphase. FIGURE 21. Longitudinal section through an early ciliated stage, approximately three hours, showing central mound of cells at the animal pole, two of the four vacuolated plates of cells, and small round body (probably a polar body) beneath membrane at the right.



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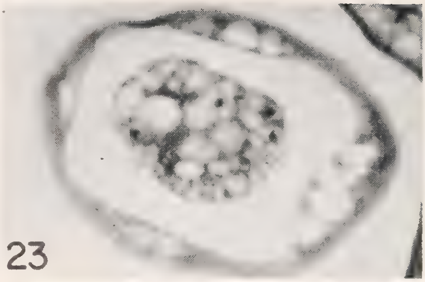

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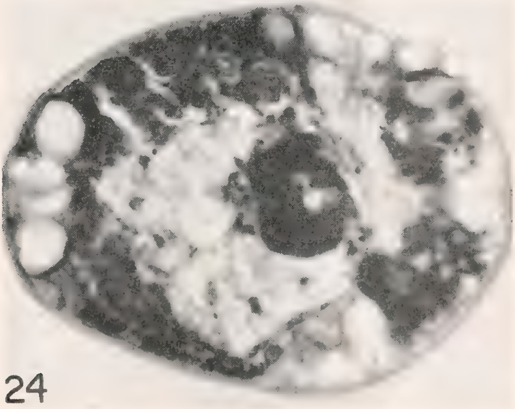

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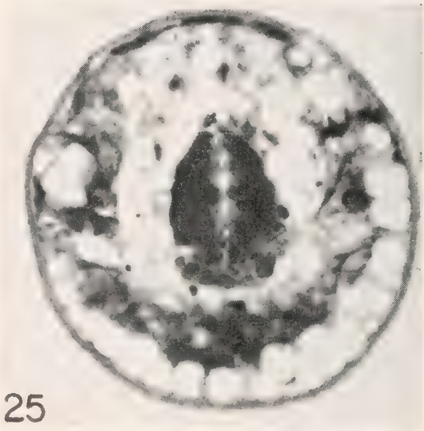

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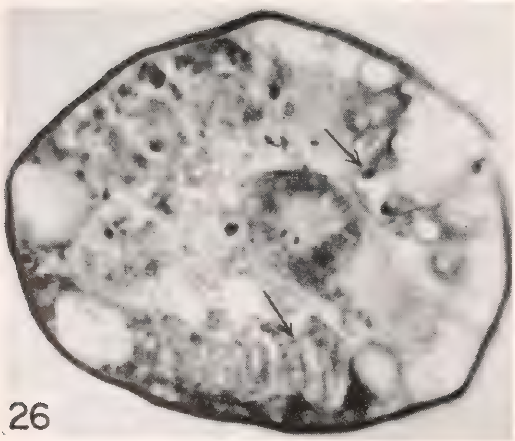

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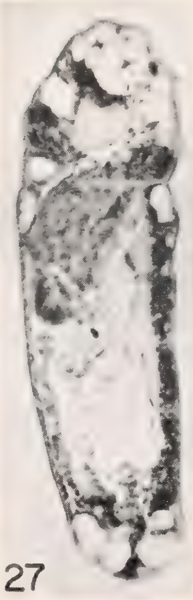

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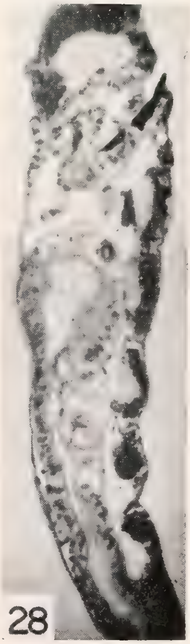

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Plate IV

continuous with the droplet-filled darker mid-gut region (Fig. 11, droplets not in focus). The arms of the Y-shaped mid-gut surround the pharynx. The thick-walled, rather transparent hind-gut, presumably ectodermal, is forming. In some batches, buds of the three more dorsal tentacles are obvious, as well as the rudiments of the two anal cirri.

Further internal structure can be seen in serial sections. Figure 27 is a sagittal section of this stage, showing pharynx and incipient jaws, narrow esophageal portion, and the mid-gut which has no lumen as yet and contains some dark pigment spots. A coelom has appeared, two flattened nuclei of the ventral peritoneal cells being clearly visible. The ventral body wall is thick compared with the dorsal and a ventral nerve cord is differentiating just beneath the peritoneum. A cerebral ganglion is visible just anterior to the pharynx. At least four large posterior vacuoles are visible.

Larvae of 4 days. By this stage four sets of setae are visible externally and a fifth is beginning to form internally. The apical tuft was not observed and proto- and telotrochs are reduced. A few superficial scattered dark pigment spots can be seen in living larvae, and the endodermal and mid-gut contains some pigment. The transparent hind-gut has a narrow lumen. In most larvae, three well developed tentacular protrusions have appeared (Fig. 13) and buds of the two more ventral tentacles, as well as two anal cirri. Also visible through the body wall are the developing jaws (Fig. 13).

Larvae of 4 days, 8 to 12 hours. Larvae of this stage have settled on the bottom and some may be observed in transparent slime tubes. They have four sets of functional setae externally with a fifth beginning to protrude in some. The presetal and postsetal lobes on the parapodia of the first setigerous segment are retained in this stage and in the subsequent stages described (compare Fig. 33). Five occipital tentacles are present, one mid-dorsal, two dorso-lateral, and two ventro-lateral ones, the last two being shorter. Two anal cirri are represented by

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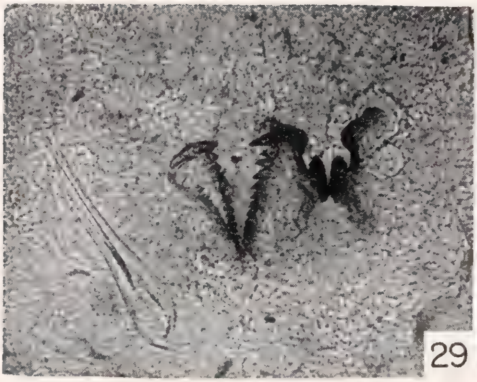
PLATE IV

EXPLANATION OF FIGURES

FIGURE 22. Frontal section of 24-hour trochophore (anterior at right) showing pharynx near center, light undifferentiated yolk mass just posterior to it, and mesodermal bands flanking the mid-gut. FIGURE 23. Transverse section through the central mound in a larva similar to that in Figure 21, showing the four plates of vacuolated cells surrounding the mound. FIGURE 24. Frontal section through a 36-hour larva (cut at 15 micra) showing pharynx (note anaphase), light undifferentiated yolk mass, and four prominent posterior vacuoles. FIGURE 25. Transverse section through the pharynx of a larva that is similar to Figure 24, showing peripheral vacuolated cells and the cilia penetrating the larval membrane. FIGURE 26. Frontal section through a 36-hour larva (cut at 10 micra) showing the pharynx (note anaphase), yolk mass, and two large posterior vacuoles. Two sets of internal setae are forming (tip of lower arrow) and two of the mucus-secreting cells with basal nuclei are visible (tip of upper arrow). FIGURE 27. Sagittal section through larva of 3⅓ days, with four sets of setae. The jaws are beginning to form in the pharynx, the cerebral ganglion (light area) is anterior to them, and the mid-gut (without a lumen) is posterior to them. Note also the posterior vacuoles, the coelom around the gut, and the peritoneal cells (two nuclei clear) lying in contact with the ventral nerve cord. The ventral body wall is thicker than the dorsal. FIGURE 28. Sagittal section through larva of 5½ days, with five sets of setae. The same structures seen in Figure 27 may be noted, although they are more highly differentiated. The mid-gut region now has a lumen continuous with the intestine which opens by way of a ventral anus, and some of the mid-gut cells have black pigment.



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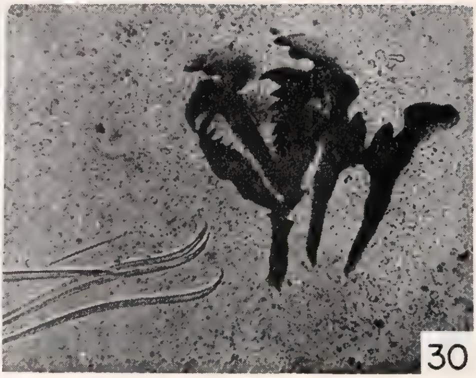

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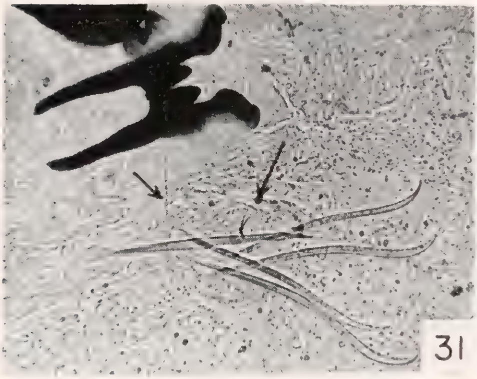

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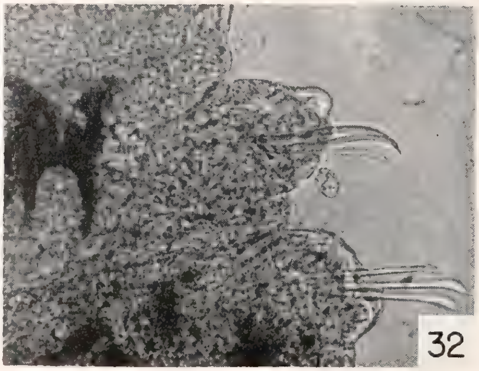

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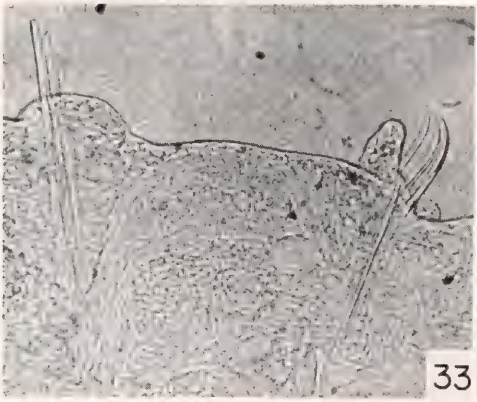

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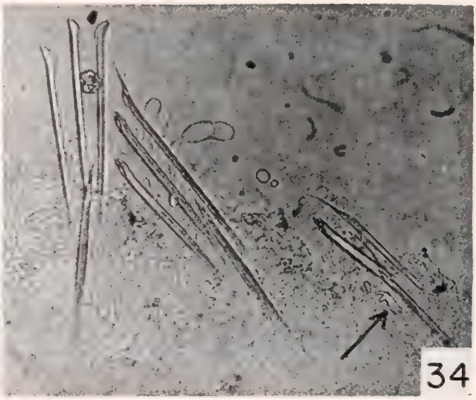

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PLATE V
EXPLANATION OF FIGURES

FIGURE 29. Differentiating jaws of a larva of 4⅓ days showing toothed asymmetrical maxillary plates on the left (an extra toothed portion is present on the left side) and mandibles on the right. Note also the bundle of curved pointed setae from the first setigerous segment.
FIGURE 30. Jaws from a larva of approximately eleven days, showing further differentiation

buds in some larvae of this stage, but are more obvious in others. Tufts of cilia, visible at the eye level in some, probably represent the remains of the prototroch. A prominent telotroch is still present. Also visible externally are jaws consisting of asymmetrical maxillary plates with well defined teeth and differentiating mandibles (Fig. 29). An esophagus is differentiating between pharynx and mid-gut, and the latter continues posteriorly into the hind-gut. The dark yolk mass and droplets are restricted to the mid-gut and black pigment is visible in its lining. Some of the larvae appeared to be feeding on microorganisms.

Larvae of 5½ to 7½ days. Larvae of 5½ days have 5 sets of functional setae although the last set is usually not completely formed; in some cases a sixth set is differentiating internally. Some larvae may be observed in transparent slime tubes on the bottom, and in one instance a larva was observed turning around in its tube. Larvae which have not formed tubes often stick to the bottom at this stage and may constrict in two in attempting to free themselves. The five occipital tentacles are "knobby" and well developed (Fig. 15): the three more dorsal ones are approximately 150 micra in length and have two basal segments by 7 days; the two more ventral ones are shorter and have one basal segment each. Two anal cirri are well developed (approximately 30 micra in length) and "knobby" (Fig. 15).

A number of the differentiating internal structures of this stage can be illustrated by Figure 28. This is a sagittal section through a larva with 5 sets of setae (5½ days old) and with well developed jaws associated with the pharynx. The mid-gut is patent throughout, its lumen being continuous with that of the hind-gut which, in turn, opens ventrally through the anus. The coelom has enlarged as compared with the preceding stage (Fig. 27). Nuclei of two of the flattened peritoneal cells are visible ventrally (the peritoneum can also be seen in living larvae), and the cerebral ganglion and ventral nerve cord are clearly visible.

Larvae of 8 days, 8 hours and older. By 8⅓ days, 6 sets of setae have formed externally in most cases and are complete, or almost so. However, some larvae take one to three days longer to form the sixth set (a few take even longer). The black jaws are well differentiated and active at these stages. The asymmetrical maxillary plates have a medial toothed margin in each half (as well as the toothed

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as compared with Figure 29. The bundle of curved pointed setae from the first setigerous segment and an additional slender rod are also visible. FIGURE 31. Curved pointed setae on the first setigerous segment of a larva of 8½ days, with six sets of setae. Characteristically, four such setae are present but here the curved tip of a fifth set is appearing (off tip of right-hand arrow). Note also the aciculum with a deeper origin than the external setae, and the slender rod (off tip of left-hand arrow). FIGURE 32. Two anterior parapodia in a larva of approximately 5 days, with four sets of setae. The curved, pointed, claw-like setae of the first setigerous segment are visible; contrast these with the short-tipped winged capillary type (one in focus) characteristic of the second, third, and fourth setigerous segments. FIGURE 33. Parapodia of first and second setigerous segments (anterior at right) in a larva of 5⅓ days, with a small fifth set of setae. The finger-like postsetal lobe and the smaller presetal lobe which are characteristic of the first parapodium are visible. FIGURE 34. Setal types from the fourth, fifth, and sixth setigerous segments (anterior at left). Note the three short-tipped winged capillary setae (and basal aciculum) characteristic of the second, third, and fourth setigerous segments, the two bidentate acicular setae and one long-tipped winged capillary seta (and basal aciculum) characteristic of the fifth, sixth, and seventh setigerous segments. The two-pronged tip (off tip of arrow) of the second bidentate acicular seta developing in the sixth setigerous segment is also visible.

additional piece; see Figure 30) and work in scissors-like fashion with the mandibles either held stationary or with both jaws working alternately in an antero-posterior direction. The maxillary plates move forward, open, and then close during their posterior movement.

In a few cases a culture of algae was allowed to accumulate in the stender dishes. The larvae in these cases appeared to be feeding on the algae although the mid-gut was still dark with stored food material and contained large food vacuoles. The larvae upon occasion will eat their own kind as in one instance black jaws of another larva were observed in the mid-gut of an 11½-day larva, and one larva appeared to be "gnawing" on another living larva stuck to it. An active rolling movement from side to side was noted in the esophageal region of a number of larvae, and in one food particles were noted in this region of the fore-gut which is very thick-walled.

The five occipital tentacles are similar to those of the preceding stage except that they are longer, the dorsal ones measuring approximately 225 micra in 9-day larvae. Anal cirri in larvae of this age are approximately 50 micra long.

Headless larvae, capable of moving about, were observed occasionally. Larvae of this age tend to stick to the bottom of the dish, often on their backs, in which case they may constrict in two in an attempt to become free.

The larvae were not fed (except for any microorganisms which came through the sand-filtered sea water) and may live as long as the yolk material lasts in the mid-gut (this area becomes transparent when the food supply is gone). Over several summers, 6 sets was the maximum number of setae observed in these larvae of *D. cuprea*. However, in the summer of 1958, 7 sets were recorded for nine larvae, in two (from different batches) by 13½ days of development, in one by 14½ days, in two (from different batches) by 17½ days, and in one by 18½ days of development. One larva from this last batch did not develop a seventh set until the twenty-fifth day, and another from this batch until the thirtieth day of development. One from a different batch developed a seventh set by the twenty-sixth day. Among these larvae the oldest lived for 13 days after developing a seventh set of setae, dying at an age of 30½ days. Most larvae died before developing a seventh set. The types of setae are described in more detail below.

Types of larval setae and their order of appearance. By the time 5 sets of setae have formed in these larvae, four types of setae have differentiated. The type (or types) and distribution of each are characteristic for each segment. As indicated in Figures 29 to 34, those in the first setigerous segment are different from any of the others, those in segments two, three and four are similar, and those in segment five are new types which are retained in segments six and seven. One aciculum is associated with each setigerous sac at all levels. These internal basal setae have a deeper origin than the others (Figs. 31 and 34) and appear to direct the movements of the external ones. Once the direction of movement has been determined at any one level, the external setal complement seems to work against the aciculum which thus acts as a fulcrum.

The following tables indicate the setigerous segments, the number and types of setae in each setigerous sac (omitting acicula which are present at all levels), the time of appearance at each level, and the setal complement of each segment at successive developmental stages. Photomicrographs are presented to help in the

TABLE I

Time of appearance of setal types in various segments

Setigerous segment	Type of setae	Time of external appearance
1	3C	2 days
	3C + tip of C	$3\frac{1}{2}$ days
	4C	$4\frac{1}{3}$ days
2	2S	2 days
	3S	$2\frac{1}{2}$ days
3	2S	$2\frac{1}{2}$ days
	3S	$3\frac{1}{2}$ days
4	2S	$2\frac{1}{2}$ days
	3S	$3\frac{1}{2}$ days
5	1B, 1L	$4\frac{1}{2}$ days
	2B, 1L	$5\frac{1}{2}$ days
6	1B, 1L	7 days
	2B, 1L	$8\frac{1}{2}$ days
7	1B, 1L	13 days (typical?)
	2B, 1L	?

identification of these setal types. The key to the letters in the tables is as follows: C—curved pointed type (Figs. 29 to 33), S—short-tipped winged capillary type (Figs. 32 to 34), B—bidentate acicular type (Fig. 34), L—long-tipped winged capillary type (Fig. 34).

The individual setae develop in a disto-proximal direction, the tip differentiating first. This was observed repeatedly in "dry" mounts. For example, in the first setigerous segment of a 4-day larva, three curved setae are complete and just the curved tip of the fourth is visible internally. In the fifth setigerous segment of 4- to 6-day larvae, one of the bidentate setae and the aciculum appear to develop simultaneously; then the long-tipped seta of this level develops and before it is completed the two-pronged tip of the second bidentate seta has developed internally (Fig. 34). This sequence of setal development noted in setigerous segment number five is followed also in the sixth and seventh segments.

In one larva ($8\frac{1}{2}$ days old) the distal tip of a fifth seta of the curved type characteristic of segment 1 was noted (Fig. 31). This indicates that 4 curved setae may not be the full complement for this level; however, this one case may not represent the typical condition. Also, in a number of larvae of 8 days, 8 hours

TABLE II

Distribution of setal types by segments at different stages

Larval stage	Setigerous segment						
	1	2	3	4	5	6	7
3 parapodia	3C	3S	3S				
4 parapodia	3C	3S	3S	3S			
5 parapodia	4C	3S	3S	3S	2B, 1L		
6 parapodia	4C	3S	3S	3S	2B, 1L	2B, 1L	
7 parapodia	4C	3S	3S	3S	2B, 1L	2B, 1L	2B, 1L

and older, a tiny slender rod was noted in both of the first setigerous sacs (Figs. 30 and 31). Its presence was not observed consistently throughout this age group.

As suggested by the tables, the setae once formed were retained throughout the period of observation. This is in contrast to Wilson's analysis of the succession of larval bristles in *Nereis pelagica* (1932) in which he found that as successive setae formed, the ones more anterior began falling out.

DISCUSSION

Certain aspects of the development of the egg and of the early larvae of *Diopatra cuprea* seem to be peculiar to this species, and in other instances to this genus or to the closely related genus, Onuphis. The curious process by which the eggs are formed in the ovary has been described by Andrews (1891b) and recently has been briefly reviewed by Costello *et al.* (1957). Lieber (1931) has described this process for *D. amboinensis.* Andrews (1891b) suggests that the algal-like strings of "nurse" cells attached to the developing egg may have a supportive function while the eggs are floating free in the coelom, rather than a nutritive one. However, Treadwell (1921, page 81) states that in the eggs of *Diopatra cuprea* at Woods Hole he was able to demonstrate a "definite communication pore between the ovum and the first cell of the chain, indicating that they are true 'nurse' cells." Lieber (1931) in a detailed study of oogenesis in Diopatra described and figured a cytoplasmic connection between the developing egg of *D. amboinensis* and its attached "nurse" cell and concluded that the cells were, in fact, nutritive in function and, therefore, properly termed nurse cells. The communication pore noted by Treadwell (1921) may conceivably represent the area where an amoeboid process of the egg could contact the cytoplasm of the "nurse" cell.

Lieber (1931) has described a micropyle in the egg membrane of *D. amboinensis.* The defect observed near the vegetal pole in some eggs of *D. cuprea* in the present investigation may be a micropyle, although Andrews (1891b) makes no mention of it in either *D. cuprea* or *D. magna.* These defects may instead represent the remains of the communication pore noted by Treadwell (1921) in the developing oocyte.

It has been noted that the ripe eggs of *Diopatra cuprea* appear to be perforated. The canalicular nature of the membrane has been demonstrated in stained eggs of Diopatra by Lieber (1931). A porous membrane is not restricted to the eggs of Diopatra but has been noted in other polychaete eggs, for example, those of *Arenicola cristata* (Wilson, 1882).

Retention of the egg membrane as a larval cuticle (noted in *D. cuprea*) apparently is not uncommon among polychaetes. Wilson (1882, page 295) states, "The persistence in some cases of the chorion as the larval cuticle is a remarkable occurrence entirely confined, so far as known, to the Chaetopods and Gephyrea, and by no means universal among them." Examples of species which retain the original egg membrane are *Clymenella torquata* and *Arenicola cristata* (Wilson, 1882), *Nereis diversicolor* (Dales, 1950), and *Tharyx marioni* (Dales, 1951).

The four anterior vacuolated plates of cells which have formed by the time ciliation has been attained are peculiar to this form insofar as the writer knows, and appear to originate from the four groups of prototroch cells.

The significance of the curious arrangement of yolk spheres into peripherally located yolk plates has not been determined, for the main mass of yolk remains in the central endodermal position (mid-gut region) of the trochophore. One possibility is that these peripheral plates may serve as a more efficiently placed food supply for the rather precocious development of the setae and associated muscle strands which differentiate from the mesoderm just medial to them.

As has been noted in the introduction there seems to be considerable confusion in the literature concerning the identification of larvae and earlier stages ascribed to *Diopatra cuprea.* It is well known that larval types are difficult to identify. Two important characteristics used for distinguishing between larvae are the jaws and setal types. The conspicuous asymmetry of the maxillary plates in *Diopatra cuprea* has been noted (Figs. 29 and 30). Monro (1924), in his description of the post-larval stage of *D. cuprea,* also pictures the unpaired, toothed plate associated with the otherwise symmetrical maxillae. This asymmetrical jaw type is characteristic of adult onuphids and eunicids. The functional significance of unpaired maxillary plates in otherwise symmetrical jaws, which appear to work in scissors-like fashion, is obscure. Comparing the diagram of the upper jaw pictured in Monro (1924, Fig. 6, page 197) with the writer's photomicrograph of the jaws of an 11-day larva (Fig. 30), one may conclude that they are closely similar and in all probability could have come from larvae of the same species when one considers the difference in age. Monro (1924) includes a brief discussion of the possible evolution of jaws within the eunicids and closely related groups.

Setae develop precociously in *Diopatra cuprea,* at least as compared with some of the nereids, such as *Nereis pelagica* (Wilson, 1932) and *Nereis diversicolor* (Dales, 1950). The importance of setal types in distinguishing between larvae is indicated by the work of Wilson (1932), Krishnan (1936), Dales (1950), *et al.* A comparison of the setae pictured here with the description and diagrams in Monro's post-larval stage (1924) suggests that the larvae described by Monro belong to a closely related species, if not to *D. cuprea.* Development of the first setigerous segment (Monro, 1924, Figure 2, and text, page 195) is in agreement with the findings described in the present study, but Monro indicates that from the second through the fifth set all setae are of the short-tipped winged capillary type. The view pictured is not clear (Fig. 3, page 195), and this setal type may or may not fit the type shown in the present investigation (Figs. 32, 33, and 34). In contrast to Monro's larvae, the fifth set of setae observed in the present study has a new setal complement which includes a bidentate acicular type which is retained in segments 6 and 7 (Fig. 34). Beginning on the sixth segment of Monro's larvae a setigerous type (Fig. 4, page 196) appears which probably could be developed from the bidentate acicular type described here (Fig. 34) by the development of a hook. However, to be comparable to the larvae described by the writer, this hooked type should begin on the fifth parapodium instead of the sixth. Thus, the two species may not be identical.

Wilson (1882) describes and figures some early stages in the development of a polychaete which he identifies as *Diopatra cuprea.* These larvae, however, were obtained from gelatinous egg masses, and Andrews (1891a, 1891b) states that these early stages and larvae described by Wilson do not belong to *Diopatra cuprea* but to *Diopatra magna.* Monro (1924) notes that Andrews does not give the basis for his statement and Monro, therefore, questions its validity. Treadwell (1921) has shown that the polychaetes described in the literature as *D. magna* in reality belong to another genus which he has designated as Onuphis. Both Diopatra and Onuphis are now accepted as distinct genera although they are closely related ones (Dr. Marian H. Pettibone, personal communication; also see Hartman, 1945, page 24, and Hartman, 1951, page 51, for keys separating these two genera). Treadwell (1921) further points out the possibility that the larvae described by Wilson are really those of *Onuphis magna* and seems inclined to agree with Andrew's interpretation. A comparison of the ciliated larva pictured by Treadwell from the gelatinous egg masses of *Onuphis magna* (1921, Plate 7, Figure 5) with that figured by Wilson (1882, Plate XXIII, Fig. 10) shows more similarity between these two larvae than between Wilson's larvae and those of *D. cuprea* described in the present study.

Comparing Wilson's larvae with the larvae pictured here, raised from the fertilized eggs of *D. cuprea*, certain differences are noted. No stages in the present study were observed that were as pear-shaped as Wilson's Figures 89 and 90 (Plate XXI), nor was any stage observed so markedly spotted with pigment as the larva in Wilson's Figure 89. Further, the rudimentary apical tuft shown is in marked contrast to the prominent apical tuft in the larvae here described. A comparison of larvae with five sets of setae shows that there are differences between those of Wilson (1882, Plate XXIII, Fig. 10, and description on page 289) and those pictured and described by the writer. In *Diopatra cuprea*, in the present study, no dorsal cirri were observed, five occipital tentacles are present in normal larvae at this setal stage, and the mid-dorsal tentacle is almost the same size as the dorso-lateral (contrast Wilson's Fig. 10, Plate XXIII). Also a clearly defined pharynx and well developed jaws are visible at this stage (Figs. 14 and 15 of the present paper; however, Wilson and Treadwell may have intentionally omitted internal structures from their drawings). Further, the enlarged tip of the one setal type shown in Wilson's larva (Plate XXI, Fig. 91) is different from any here described for *D. cuprea* (Figs. 31 and 34), although it is possible that this type might develop in a later stage.

Distribution of the two species in question provides further evidence concerning the possibility of erroneous identification of their larvae. Both *Diopatra cuprea* and *Onuphis magna* are found intertidally in the Beaufort, North Carolina, area (Hartman, 1945) and in the Gulf of Mexico (Hartman, 1951); there is, therefore, a chance of confusing the egg cases of the two genera in these areas. Thus far, however, *D. cuprea* is the only onuphid found intertidally in the Woods Hole area (Dr. Marian H. Pettibone, personal communication), so to date there is no possibility of confusion between these two onuphids (*D. cuprea* and *O. magna*) in the intertidal zone at Woods Hole. The writer is led to the conclusion, therefore, that the stages pictured by Wilson do not belong to *Diopatra cuprea* and probably belong to *Onuphis magna* (*D. magna* of Andrews) as Andrews has stated.

If Andrews is correct—and the evidence presented here indicates that he is—then the gelatinous egg masses found by Wilson belong to *Onuphis magna*. Insofar as the writer knows, gelatinous egg masses of *D. cuprea* have never been found in the Woods Hole area where this species is common. She herself has never observed them and Mr. Milton B. Gray, who has collected *D. cuprea* for a number of summers in the Woods Hole area (both for investigators and for Course work), has never seen them (personal communication). Circumstantial evidence presented by Monro (1924) indicates that the eggs of *D. cuprea* are laid inside the tube (where the larvae develop) rather than in gelatinous egg capsules lying free on the sand. However, the possibility remains that Monro is not dealing with *D. cuprea* but with a closely related species. The one time normal spawn jelly was observed in the present study, it dissolved readily in sea water. This property of the jelly and the facts that cilia develop early and that the larva forms a prominent apical tuft suggest that *D. cuprea* may have a free-swimming stage.

The writer, with the above observations in mind, would like to suggest that the egg masses with developing larvae which have been noted along the Gulf of Mexico (Hartman, 1951) as well as at Beaufort, North Carolina (Andrews, 1891b; Hartman, 1945; Wilson, 1882), belong to *Onuphis magna* and not to *Diopatra cuprea*. Both species have been described as occurring together in these areas although their distribution along the Gulf of Mexico is somewhat different (Hartman, 1951).

With the confusion of these larval types apparent in the literature, the brief study of the setal types of *D. cuprea* included here may serve as at least one criterion for distinguishing between the species of onuphids in the future. The usefulness of setal types is apparent if one compares the table given by Krishnan (1936, page 521) for *D. variabilis* (Southern) with the tables included here for *D. cuprea*.

In summary, one is led to the conclusion that the early stages and larvae described by the several investigators cited probably do not belong to the species, *Diopatra cuprea*, but to a closely related genus or species, in two instances probably to *Onuphis magna* which is the *Diopatra magna* of Andrews.

Further, this would seem to indicate that the descriptions of the writer for *Diopatra cuprea* are the only ones which can be correctly attributed to this species, with the possible exception of Monro's post-larval description which may belong to *D. cuprea*. The possibility remains, however, that some investigation not here cited has escaped the writer's attention.

The problem of activation of the egg of *D. cuprea* will have to be solved before this egg can be used to any extent either for experimental purposes or for class use. Some histochemical tests have been run on these stages (Allen, 1957) and it is hoped that in working further with the eggs of *D. cuprea* some of the problems noted will be solved. Further details of development may then be worked out to serve as a basis for experimental and histochemical studies.

SUMMARY

1. Larvae of *Diopatra cuprea* (Bosc) have been raised, following artificial fertilization, to a stage with seven sets of setae. Observations on living stages and also on fixed and stained preparations have been described and photographed.

2. Cell lineage studies have not been made, but observations indicate that the early cleavages are typical of those for spiral cleavage and that the ciliated stage (age, three hours) has a typical annelid cross and apical rosette. It, therefore, seems justifiable to conclude that the development of *Diopatra cuprea* follows the typical spiral pattern and mosaic development characteristic of other polychaetous annelids.

3. Peculiarities of the development of this polychaete, and possibly of closely related species, are the following: the peculiar algal-like nurse cells attached to the developing oocyte (also characteristic of Onuphis eggs) when floating free in the coelom, the amazing rapidity of development to the free-swimming stage (three hours), the four plates of cells which appear to develop from cells of the prototroch and their peculiar posterior extensions into at least four plates of yolk spheres, and the asymmetry of the maxillary plates.

4. Very little can be found in the literature on the embryology of the genus, Diopatra, and at least two authors have pointed out the possibility of error as to species in the identification of the developmental stages. Evidence is presented here which indicates that the early embryological and larval stages described by other investigators have been erroneously assigned to *Diopatra cuprea.*

5. If the above is correct—and it would appear that *Diopatra cuprea* is the only onuphid found intertidally in the Woods Hole area—one may conclude that the investigation presented by the writer is probably the only study recorded in the literature on the early developmental stages of *Diopatra cuprea* (Bosc). This is exclusive of Monro's description of the later (post-larval) stage which, if not belonging to *D. cuprea,* is undoubtedly closely related to this species.

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A CONTRIBUTION TO THE BIOLOGY OF A DEEP SEA ECHINOID, ALLOCENTROTUS FRAGILIS (JACKSON)[1]

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In February, 1957, a hydrographic team[3] from the Hopkins Marine Station accidentally discovered a bed of *Allocentrotus fragilis* (Swann, 1953) at a depth of 68 to 98 fathoms in Monterey Bay, California. This discovery was made during a routine hydrographic run. At the time a mid-water plankton haul with a standard one-meter net was in progress. The Hopkins Marine Station research vessel, the "Tage," had apparently drifted with the onshore current. When the net was surfaced, to their surprise and delight, the team found approximately two dozen specimens of the deep sea urchin, *Allocentrotus.* This was the first time that the animal had been obtained alive and intact in large numbers. At this spot the fathometer indicated 80 fathoms and a radio "fix" recorded the position of the boat to be 36°37′54″ N and 122°01′12″ W. All subsequent hauls were started from this station.

Since a project on the biology of the shore sea urchins, *Strongylocentrotus purpuratus* and *S. franciscanus,* was in progress at the Hopkins Marine Station, the chance finding of a bed of the deep sea urchins was of immediate comparative interest. Consequently, whenever possible, studies were made on the biology of *Allocentrotus* for comparison with *Strongylocentrotus.*

The oceanographic vessel, "Tage," was used for all work reported here. For dredging a four-meter beam trawl was employed. The average dredging time was twenty minutes. The entire sample, consisting of a variety of organisms, was brought into the laboratory in live condition in a tub of sea water. The animals were sorted and placed in separate tanks of running sea water. The species were identified and at times the number of individuals counted.

The gonad index of the sea urchins, indicating the reproductive condition of the urchins, was determined as in previous studies, as were also the biochemical constituents of body fluid and tissues (Lasker and Giese, 1954; Bennett and Giese, 1955).

Habitat of Allocentrotus

Some of the physical features of the habitat of *Allocentrotus* should be considered in order to gain an understanding of the conditions under which this species

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[3] Under the direction of Professor R. L. Bolin of the Hopkins Marine Station and including Mr. Thomas Fast and Mr. Robert Aughtry operating with the financial assistance of Grant N60NR-26127 and Grant NSF-G-1780.

lives in this area in Monterey Bay. By systematic grid dredging, the area of the sea urchin bed was estimated to be about one square mile. The depth of the area in which the urchins were taken varies between 55 to 90 fathoms, the shallow part of the bed lying on the continental shelf, the deeper part bordering the Monterey Canyon.

Dredges at various depths indicate that the larger animals tend to inhabit the deeper regions near the Canyon, whereas the smaller animals are more frequently found in shallower areas. These results are summarized in Table I.

The area nearest the Canyon is relatively flat and is composed of gravel and sand overlying gray silt (Galliher, 1932a, 1932b). From time to time, however, large boulders mainly of granite and shale, the largest of which weighed approximately 15 kilograms, were brought up in the dredge. In the shale young urchins were frequently observed in their burrows, as illustrated in Figure 1E. As the shoreline is approached the configuration of the bottom is somewhat changed, consisting mainly of granitic rock and coarse sand.

Table I

Sizes of Allocentrotus taken at various depths

Bathymetrical range in fathoms	Range in size of test diameter* in mm.
55–65	11.2– 21.3
60–65	11.2– 18.0
68	13.3– 29.4
65–90	55.0–103.3

* The measurement was made across the widest part of the test (the ambitus).

Olga Hartman (1955) has published a photograph of *Allocentrotus* taken at 350 to 400 fathoms in the San Pedro Basin 11 miles northeast of Avalon, Catalina Island, California. It was found in a sandy mud which appears to be relatively flat except for small mounds.

As this species has been taken from 48 to 417 fathoms (Clark, 1912), the data considered in this paper represent only a limited aspect of the habitat of *Allocentrotus*. It is possible that for the larger range over which it occurs, physical conditions other than those described above may obtain.

Animals associated with Allocentrotus

Since the organisms found in the same habitat as *Allocentrotus* may play a role in the ecology of the species, all of the organisms which came up in the beam trawl were identified when possible and counts of their numbers were made to ascertain their relative abundance. These organisms are listed in Table II. It is observed that protozoans, coelenterates, annelids, nematodes, mollusks, arthropods, echinoderms and fishes are found in the association. The interrelationships between the various forms have not been studied.

Because of the random nature of the sampling it is difficult to say much about the relative abundance of the various species in the natural habitat. However, the crab, *Mursia*, is usually obtained, sometimes in large numbers as is the holothuroid, *Stichopus* and an unknown tectibranch. The starfishes *Mediaster, Pycnopodia,*

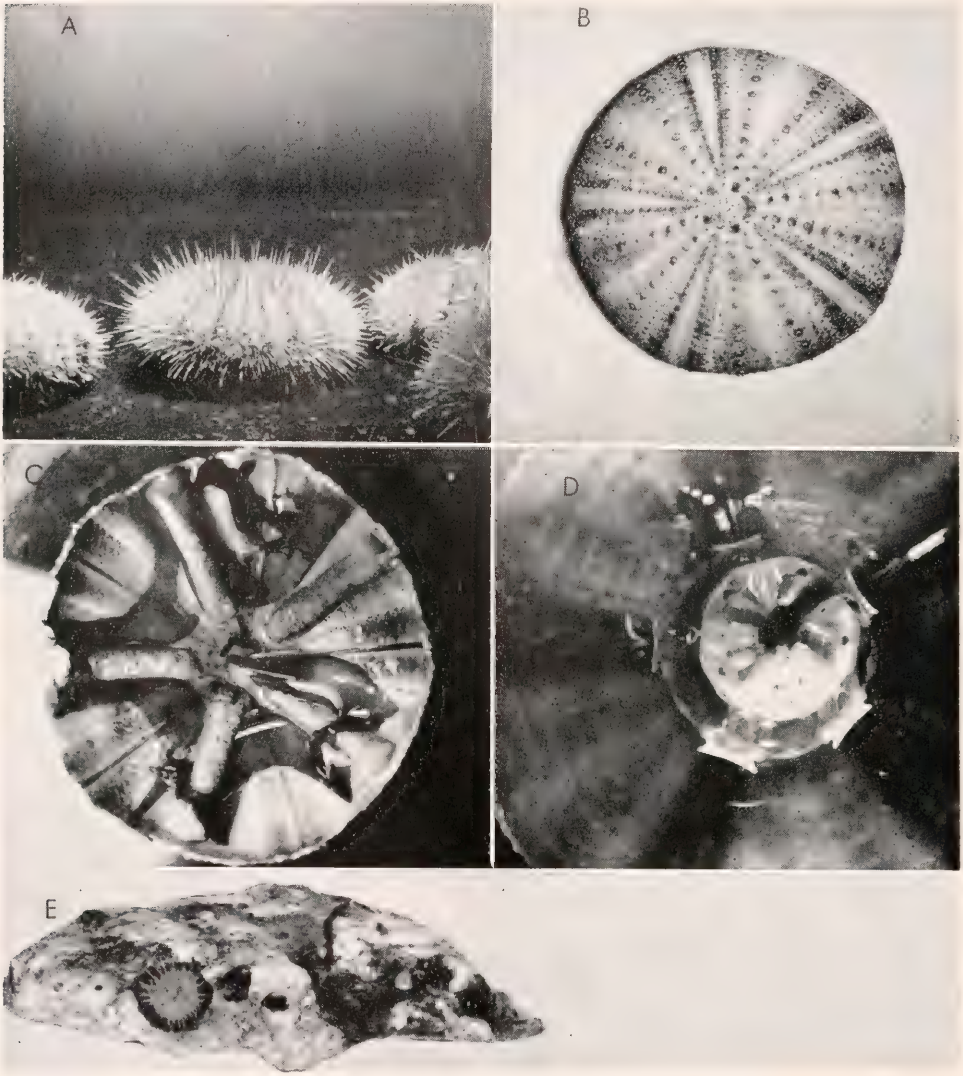

FIGURE 1. A, An adult *Allocentrotus fragilis* 67 mm. in diameter. B, A test of *Allocentrotus fragilis* 73 mm. in diameter. C, A photograph of the aboral half of the shell of *Allocentrotus* showing the gonads. D, The Aristotle's lantern and the peripharyngeal coelom of *Allocentrotus*. E, A specimen of *Allocentrotus fragilis* (15 mm. in diameter) imbedded in its shale burrow.

Henricia, *Pteraster* and *Astropecten* are also rather likely to be among the specimens brought up in the trawl. From the numerous species and their relative abundance it seems likely that the habitat of *Allocentrotus* is one with relative abundance of food.

Olga Hartman (1955) found *Allocentrotus* in deep waters (350–400 fathoms) in association with a variety of animals (legend to plate 2A): "A two-foot square sample from the bottom yielded glass sponge, many foraminiferans, 20 or more species of annelids, many ophiuroids, and a large percentage of new or little known animals." In her photograph of the benthos a crinoid and a sea star are seen among the numerous *Allocentrotus* which appear to be spaced about a meter from one another.

It is of interest to note that a rhabdocoel parasite similar to *Syndesmus franciscanus* commonly found in the shore urchin (Lehman, 1946) was observed in the gut of several specimens of *Allocentrotus*, and the specimens are of the same size as those found in *Strongylocentrotus*. One, two or three at most, were found in the gut and the incidence of infection was low.

Table II

Animals taken in association with Allocentrotus fragilis

Protozoans
  Foraminiferans

Coelenterates
  Psammogorgus
  Metridium senile

Annelids
  Three different species of polychaetes

Nematodes
  A variety of specimens

Mollusca
  Rosea pacifica (octopus)
  Numerous unidentified small gastropods

Arthropods
  Crustaceans
  Munidopsis sp.
  Spirontocaris sp.
  Mursia quadichaudii
  Paguristes sp.

Echinoderms
  Asteroids
  Mediaster aequalis
  Pycnopodia helianthoides
  Pteraster tessalatus
  Henricia aspera
  Orthasterias koehleri

Echinoderms
  Stylasterias sp.
  Astropecten californicus
  Luidia foliolata

Ophiuroids
  Gorgonocephalus eucnemis
  Two other species of brittle stars

Holothuroids
  Stichopus californicus

Vertebrates
  Fishes representing the following families:

  Liparidae
  Agonidae
  Zoarcidae
  Ophidiidae
  Cottidae
  Batrachoididae
  Scorpaenidae
  Bothidae
  Pleuronectidae
  Petromyzontidae
    Entophenus tridentatus
  Rajidae
    Raja sp.
  Chimaeridae
    Hydrolagus colliei

Nutrition of Allocentrotus

Although the *Allocentrotus* bed occurs in the euphotic zone (down to 200 meters according to Sverdrup *et al.*, 1942), no conspicuous algae have ever come up in our numerous dredgings. The large algae serve as the main food of the shore urchins of the genus *Strongylocentrotus* (Lasker and Giese, 1954; Bennett and Giese, 1955). The sediments collected along with *Allocentrotus* in the dredge hauls consist of a variety of decomposing organic materials in which strands of algae, diatoms, sponge spicules, nematodes, foraminiferan and other shells, as well as other protozoans are found among numerous bacteria. Sometimes live nematodes and protozoans were observed in the mud.

The gut usually contains numerous olive-green pellets measuring 1.2 to 2.8 mm. in diameter, relatively compact but soft in texture. When these pellets are crushed and examined microscopically they are found to contain many small glassy rings (desmids?), foraminiferans, sponge spicules, a variety of diatoms, sand particles and unidentifiable organic particles. Acidification with HCl indicates that most of the skeletal particles are silicious since they do not dissolve. Treatment with concentrated HNO_3 oxidized all the fluffy organic material leaving the silicious diatom skeletons, sponge spicules and glassy rings. In the collection of July 25, 1958 the intestines of all the animals sampled were more completely filled with pellets than in the other collections. The pellets were, in addition, a more vivid green than in all the other cases. Extracts indicated the presence of a brown pigment, fucoxanthin, plus a large amount of chlorophyll. The feeding was correlated with a rich plankton bloom in the surface waters nearby. In the collection made on August 14, 1958, some reddish pellets consisting entirely of organic debris and bacteria were found among the green ones. The constituents of the gut pellets are shown in Figure 2.

Specimens of *Allocentrotus* which survive the hazards of the trip to the surface and arrive at the laboratory in good condition remain alive for many days. When the animals are kept out of water for even a brief time they lose body fluid and air is trapped inside the test, after which they float and die. Normal animals move about the aquaria like *Strongylocentrotus purpuratus,* though less actively, and they adhere less firmly so that they are more readily knocked off by even a small push. They right themselves much more slowly than the purple sea urchin. Attempts were made to feed *Allocentrotus* with boiled potatos, *Phyllospadix* (eel grass) and the algae, *Ulva, Iridaea,* and *Gigartina,* as well as with animal matter such as crushed mussel (*Mytilus*) and crushed deep sea crab (*Mursia*) after several days of fasting. The animals nibbled at some of the algae and at *Mytilus* and *Mursia,* dropping the material after a while, then going down to the bottom of the aquaria to nibble again. It would appear, therefore, that *Allocentrotus* is more selective than *S. purpuratus,* which eats almost any organic material when hungry and shows sustained intake for hours. However, it must be remembered that the specimens are being tested at sea level and at about 15–16° C. whereas they come from a deep sea environment where they are subjected to about 15 atmospheres of pressure and temperatures of about 9° C. It is difficult to say what their behavior might be in their natural environment.

It has been shown that the gonads of a purple sea urchin are probably the main storage organs of the animal, the gonads in a gravid animal increasing to a size which all but obliterates the body cavity left unoccupied by the gut and its contents. The relative mass of the gonads in gravid *Allocentrotus* is much less than that of a gravid *Strongylocentrotus.* At its peak the gonad of *Allocentrotus* is still a delicate structure, both in size and in color (pale creamy-white in the male and yellowish in the female). The gut of an *Allocentrotus* is generally well filled with pellets, but it does not appear to be as full as the gut of the two species of *Strongylocentrotus* studied. It appears, then, that food is generally less available in deeper waters

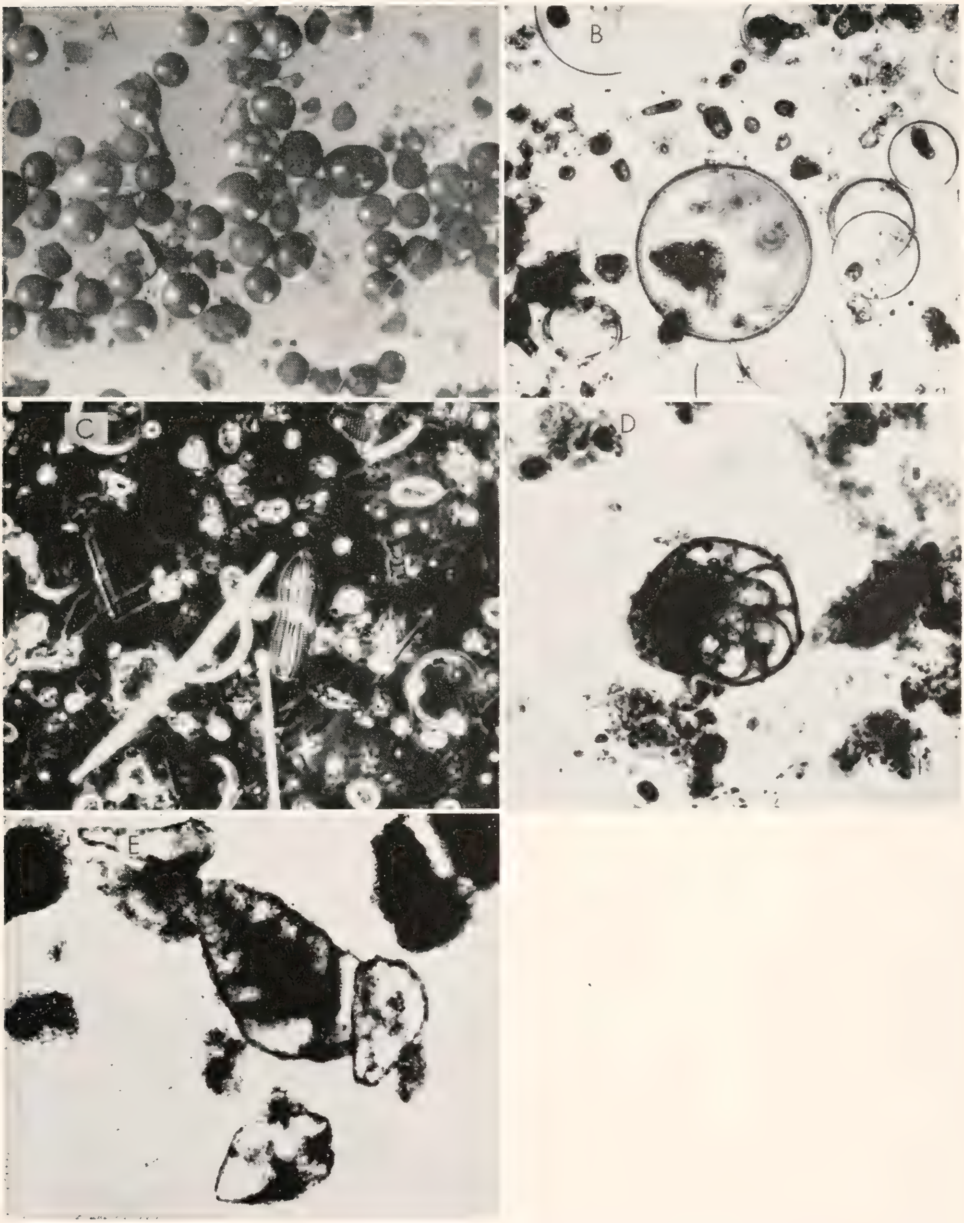

Figure 2. Food pellets of *Allocentrotus* as seen under low and high powers. A, Food pellets as removed from the intestine (× 6). B, Crushed food pellets showing desmids (× 60). C, Diatoms and sponge spicules in crushed food pellets (× 60). D and E, Foraminiferans in crushed food pellets (× 60).

than on the shore, except after an unusually rich bloom of plankton as in the collection of July 25, 1958.

Like the gonad of the two species of *Strongylocentrotus* tested, the gonad of *Allocentrotus* contains a little stored glycogen (0.36 to 0.83 per cent or an average of 0.57 per cent of the dry weight), considerable protein (about 30 per cent of the dry weight), and a large store of lipid (an average of about 28 per cent of the dry weight). The chemical constitution of the gonad of *Allocentrotus* is much like that of the gonads of other species of sea urchins although it is smaller in proportion to body size. The perivisceral fluid, which is possibly one of the channels for distribution of the food from the gut, contains nutrients in solution much like the same fluid in the other species of sea urchins tested. Total nitrogen amounted to 3.78 to 4.98 milligrams per cent, non-protein nitrogen to 1.28 to 1.34 milligrams per cent, and a small amount of lipid is present. A variety of cells is present in the perivisceral fluid, resembling those of the other species of sea urchin (Boolootian and Giese, 1958) and a clot forms much as in the other species of sea urchins tested (unpublished data).

Healthy specimens of *Allocentrotus* kept in aquaria at about 15° C. in the laboratory defecate very slowly. This may be an indication of a rather slow rate of digestion but it may be the result of the abnormal conditions in the laboratory. When animals with the gut loaded with food were brought in on July 25, 1958, they defecated copiously. Defecation may therefore depend upon how full the gut is at the time of collection.

All specimens collected sooner or later fall prey to a peculiar disorder. Small spots of dark red color begin to appear on the surface of the test. These spots then spread, covering the animal with large blotches of color. The tube feet degenerate and the spines fall off after which the animal dies. Microscopic examination of the spots indicates that they are composed mainly of dead eleocytes, the pigmented cells of the perivisceral fluid.

Reproduction

The first collection of *Allocentrotus* in February of 1957 contained individuals in full reproductive condition, the gonads of many males and females containing mature gametes in large numbers. The eggs were readily fertilized and normal development to the pluteus followed. Development was best at temperatures between 9°–14° C., cleavage being inhibited by higher temperatures.[4] The same was true for the second collection in March of 1957. However, the gonads of the animals collected in April no longer contained ripe gametes. Thereafter storms and other difficulties prevented collecting the urchins until September of 1957. The gonads of animals sampled in September, October, November and December of 1957 and in January of 1958 were well developed and of relatively large size until they spawned between January and the end of February, 1958, when the next collection was made. The gonads during the second breeding season were never as well developed as those of the first season, nor was as good a development of the embryonic stages observed.[4]

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[4] The results on development of *Allocentrotus* are being published by Dr. A. R. Moore in a separate report. We are indebted to Dr. Moore for permitting us to quote here and in footnote 6 from his unpublished data.

The reproductive state of an animal can be ascertained by measuring the ratio of the volume of the gonad to the wet weight of the animal (Lasker and Giese, 1954). This ratio times 100 has been called the gonad index. The average gonad indices determined in this manner are plotted in Figure 3. The course of the

FIGURE 3

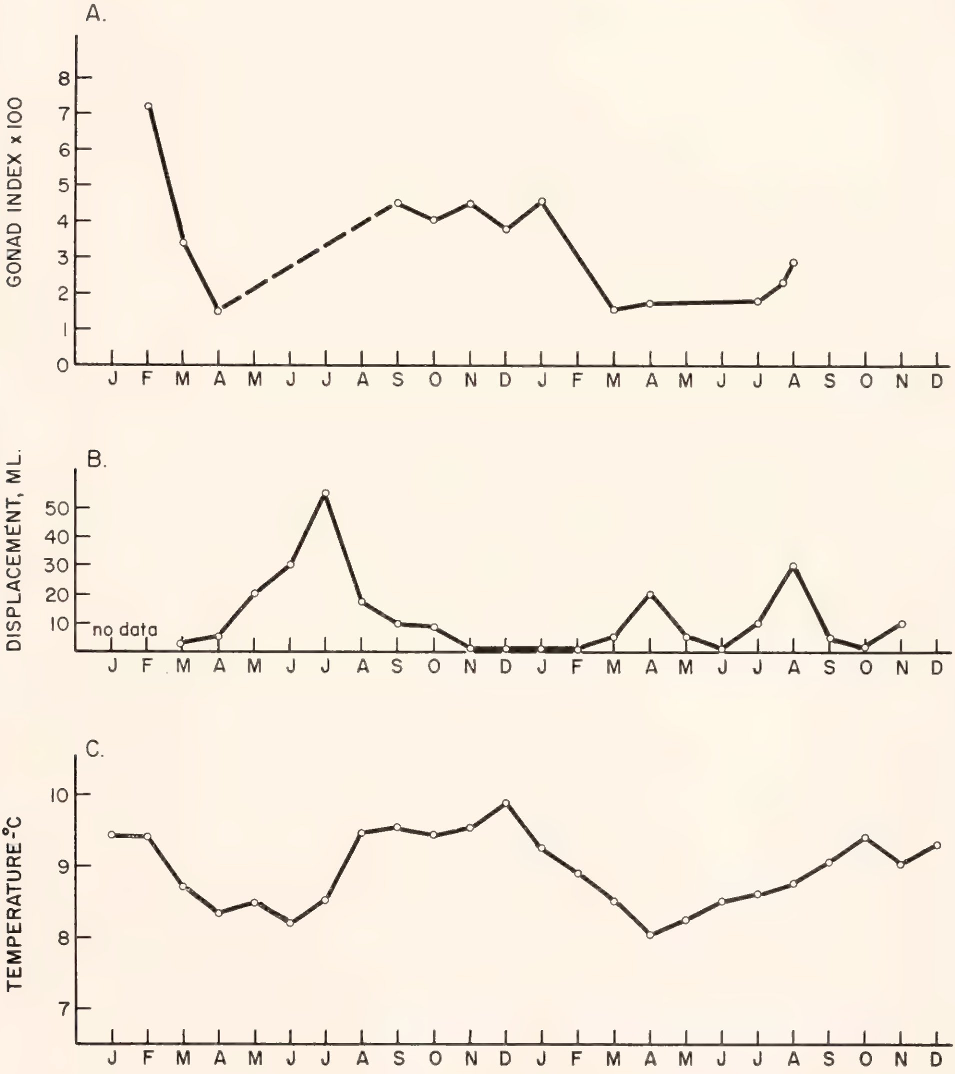

FIGURE 3. A, Gonad index of *Allocentrotus* at different times from February, 1957 to July, 1958. B, Variations in phytoplankton during the years 1954 and 1955 as determined by Barham (1957). C, Variations of thermal monthly averages between 100–200 m. as reported by Skogsberg and Phelps (1946) for the years 1936 and 1937. Same locality as that used in the present study.

curve (dashed line) from April to September, 1957 is not known but since in 1958 the gonads of animals obtained in July were just beginning to enlarge, a period of reproductive quiescence may have occurred from April to the end of June, 1957 as happened in 1958.[5]

All of the urchins used in determining the gonad index were mature, varying in wet weight from 45.5 grams to 264.0 grams and in test diameter[6] from 55.0 to 96 mm. Even a population of mature animals of similar size shows considerable variability in gonadal development at a given time. During the period when the gonads of some individuals are well developed and large, the gonads of other individuals are shrunken or undeveloped. The variability of gonad size is considerably smaller when the gonads are immature or spent.

The great variability in the gonad index during the breeding season may indicate: 1) that some individuals do not have access to adequate food to ripen or to maintain their gonads, 2) that some individuals have just spawned while others are ready to do so, or 3) that some individuals may be immature when others are gravid. A histological study of the gravid and non-gravid gonads might make it possible to decide between these alternatives.

Discussion

It is interesting to compare the biology of *Allocentrotus fragilis* to that of the intertidal sea urchin, *Strongylocentrotus purpuratus* and to that of the subtidal urchin, *S. franciscanus.* Whereas the inshore urchins generally graze on algae, *Allocentrotus* appears to graze on whatever organic material occurs in the substrate, but chiefly on organic detritus, bacteria, and microscopic animals and plants of the organic "rain." *S. purpuratus* is, on the other hand, omnivorous. When trapped in a burrow with an opening smaller than the test diameter it feeds largely on the detritus brought by sea water. In a sense, then, *Allocentrotus* represents an extension of this special feeding habit of *S. purpuratus.*

Allocentrotus lives in a community of invertebrates and fishes perhaps fewer in species and in numbers than the urchins of the intertidal and subtidal zone, although no decisive comparison can be made between the two communities because of the paucity of data for the deep sea community. It is also singularly interesting that a rhabdocoel containing hemoglobin should be present in the gut of the deep sea urchin as in the gut of shore forms.

The data gathered in 1957–58 suggest that *Allocentrotus* has an annual breeding season although the span of the cycle cannot be defined precisely at the present time. During the fall and winter months from September, 1957 to January, 1958 the gonad index remained high. In both 1957 and 1958 the gonad index fell precipitously between February and March. It is of interest to correlate 1) growth of gonads, and 2) spawning with physical conditions in Monterey Bay. Among the possible variables are 1) light, 2) temperature, 3) salinity and minerals and 4) planktonic bloom which may be correlated with upwelling.

[5] Only one *Allocentrotus* was obtained on August 14, 1957 but this male had a gonad index of 6.72 per cent, suggesting that the gonads were probably increasing in volume. Because of the general variability of size of gonads in any sample, the measurement is only indicative.

[6] The largest test diameter observed in specimens from Monterey Bay is 103.3 mm. according to Dr. A. R. Moore.

Although day-length has been correlated with breeding cycles of some invertebrates and vertebrates (Borthwick *et al.*, 1956), it does not seem likely that it is a controlling factor for *Allocentrotus* because of the low intensity of light at the depths in which this animal lives. However, some photoperiodic animals are affected by very low light intensities and to them the span of illumination is of greater importance than the intensity of the light. The possible action of light in timing the reproductive cycle of *Allocentrotus* is not excluded.

Cyclic variations in temperature of the habitat of *Allocentrotus* have been observed (Skogsberg, 1936; Skogsberg and Phelps, 1946). The data for the years 1936 and 1937 are given in Figure 3C at a depth between 100 and 200 meters. A seasonal rhythm is seen with low and fairly constant temperatures in spring and early summer. In May the temperature range at 150 meters was 8.2 to 8.5° C. in 1936, and 7.9 to 8.4° C. in 1937. In July the temperature at 150 meters began to rise, reaching a maximum by December at which time it ranged from 9.6 to 10.1° C. in 1936, and was 9.3° C. in 1937. The difference between highest and lowest temperatures is greater during upwelling of cold waters than during the period of warmer waters. The temperature variations may be correlated with three major water movements: the Oceanic period lasting from September to October, the Davidson current period lasting from November through February, and the Upwelling period occurring from late February through August. The Oceanic period and the Davidson Current generally coincide with the high thermal phase and the somewhat lower chlorinity, although chlorinity variation is never large (Skogsberg, 1936). The onset of upwelling in late February coincides with the spawning of *Allocentrotus* and may act as the trigger for initiation of the spawning. The subsequent warmer phase coincides with the period of growth of the gonads. As is to be expected, surface temperatures were found to be more variable than deep water temperatures according to Skogsberg and Phelps (1946) and the more recent CCOFI report of 1958.

The upwelling in Monterey Bay is followed by a phytoplankton bloom (Barham, 1956), as seen in Figure 3B. It is possible that the phytoplankton is used by the planktonic larvae of *Allocentrotus* and by the metamorphosed young urchins themselves when they reach the sea bottom. In this way the timing of events in the breeding cycle may ultimately depend upon the food supply, the larvae appearing at the most favorable time for their growth, namely, when phytoplankton is most abundant. All of these attempts to explain the breeding cycle of *Allocentrotus* must be considered as tentative hypotheses for which substantiating data are still needed.

SUMMARY

1. Following a chance collection of a deep sea urchin, *Allocentrotus fragilis*, from a depth of 80 fathoms, it subsequently became possible to collect the urchins on numerous occasions from the same area.

2. The area of the bed was determined by grid dredging and the nature of the habitat determined to be relatively flat, gravel and sand underlaid with gray silt containing organic detritus and microscopic organisms.

3. The deep sea urchin appears to graze on the bottom since the organisms and organic debris of the bottom sediment appear in little pellets in its gut.

4. Many types of invertebrates are associated with *Allocentrotus,* including various other echinoderms. A variety of fishes is found as well.

5. Individuals with mature gametes were obtained in February and March of 1957 and during the period of September, 1957 to January, 1958. Spawn-out appeared to occur between February and March during both years.

6. Attempts to correlate the life cycle of *Allocentrotus* with various environmental factors led to the suggestion that upwelling may trigger spawning. The planktonic larvae then presumably develop during the most effective time when the planktonic blooms occur.

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MARINE BIOLOGICAL

THE LARVAL DEVELOPMENT OF CALLINECTES SAPIDUS RATHBUN REARED IN THE LABORATORY[1]

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The crabs which comprise the family Portunidae include several commercially important species and studies on their life history have been in progress for the last 100 years. Of the British species only *Portunus puber* (L.) has been successfully reared in the laboratory through all larval stages to the first crab (Lebour, 1928). Larvae of *Carcinus maenas* Penn. have been described by a variety of workers but the complete development is not known from laboratory rearing. Of the American species *Callinectes sapidus* Rathbun is the most important commercial crab in the Western Atlantic and Gulf of Mexico. Churchill (1942) described the larval development of *C. sapidus* by reconstructing the sequence of stages from planktonic material. Hopkins (1943, 1944), rearing the larvae through the third zoeal stage, found that not all of the stages fit the description given by Churchill (1942) and was of the opinion that the larvae described by Churchill (1942) represented several different species. The complete larval development of *C. sapidus*, from hatching to the first crab stage and beyond, was first reported from laboratory rearing by Costlow, Rees and Bookhout (1959). While a brief account is given of the number of stages, the duration of the intermolt periods, and the time required for complete development, the larval stages are not described nor is detailed information given on the various environmental factors under which complete development occurred.

The present study has had two major objectives: one, to provide a detailed description of all the larval stages of *Callinectes sapidus* Rathbun reared in the laboratory; and two, to determine the effects of salinity and temperature on larval development.

METHODS

Ovigerous *Callinectes sapidus* females were obtained from the Beaufort Inlet through the cooperation of Mr. David Beveridge, captain of the commercial trawler "Beveridge." Additional females were obtained from crab pots placed in waters of lower salinity. The crabs were placed in glass battery jars containing running, filtered sea water of a salinity of 23–26 p.p.t. The battery jars were tilted so that the slight overflow passed through a series of glass trays. When the eggs hatched the larvae were carried into the glass trays by the overflow, removed by large-bore pipettes as they collected on the light side, and segregated into cultures of 50–75 zoeae per finger bowl. These were further subdivided into

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[1] These studies were aided by a contract between the National Science Foundation and Duke University, G 4400. The authors wish to express their appreciation to Mrs. W. A. Chipman and Mrs. C. King for their assistance throughout the study.

plastic compartmented boxes with one zoea per compartment. Larvae which hatched from these crabs (Series a, c, and d) were maintained at 25° C., 26.7 p.p.t. with a photoperiod of approximately 12 hours light and 12 hours darkness. The larvae which would have been designated "b" did not hatch.

To assure acclimation of the larvae to different salinities before hatching, other ovigerous crabs were placed in battery jars which did not incline but were partially filled with water of approximately the same salinity as the inlet water during the summer months (32 p.p.t.). Four salinities were obtained from the 32 p.p.t. sea water by the gradual addition of appropriate volumes of distilled water. The four salinities used were: 15 p.p.t., 20.1 p.p.t., 26.7 p.p.t. and 31.1 p.p.t. The water used for the adult crabs was aerated but not changed. The crabs were not fed and any fecal material which did appear was removed.

Some larvae which hatched at 20.1 p.p.t. were gradually changed to water of 10 p.p.t. Additional zoeae were hatched and maintained through most of the larval period at 32 p.p.t.

Table I

*Original number of Callinectes sapidus larvae maintained in 15 combinations of salinity and temperature. Because the larvae reared at 25° C., 26.7 p.p.t. were hatched from three different females at different times they are designated as a, c, and d. S—per cent survival to first crab stage; *—maintained on shaker, 120 min.*

p.p.t. / ° C.	10.5	15.6	20.1	S	26.7	S	31.1	S	32.0	S
20	108	108	108		108		108			
25	100	100	100	1.0	a) 18*	5.5	80			
	108*	108*	108*		c) 150*	2.7	150*	1.3	1000†	< 1
					d) 100	8.0				
30	108		108		108		108			

† Diluted to 28 p.p.t. on day 41.

When hatching occurred in the jars without any overflow the zoeae were removed with a large-bore pipette to finger bowls. The salinity of the water in the finger bowls was identical to the water in which hatching had occurred. Both plastic compartmented boxes and Syracuse watch glasses were used as rearing containers for larvae within each salinity. Ten zoeae were maintained in each Syracuse watch glass and 6 zoeae in each plastic compartment. Zoeae in each of the salinities were maintained at three different temperatures: 20° C., 25° C., and 30° C. Zoeae of all series were fed *Arbacia* eggs and *Artemia* nauplii which were added each day when the larvae were changed to freshly filtered sea water and clean receptacles. Some plastic boxes were maintained on an Eberbach shaker (120/min.) at 25° C. but the majority of the containers were stationary (Table I). The megalops and crab stages were fed *Artemia* nauplii plus beef liver. The compartments containing the zoeae were observed daily for exuvia and, at this time, the number of molts and the mortality were recorded.

Drawings of the zoeal stages and megalops stage were made from the exuvia of known molts and from larvae preserved at a known stage of development. All figures were made to scale on graph paper with the aid of a Whipple disc inserted in the ocular of a compound microscope. The detailed drawings of the appendages of each stage are also drawn to scale, different from that used for the whole larva, from appendages dissected out with glass needles.

RESULTS

Larval stages

First zoea: The characteristics of the first stage zoeae agree closely with those given by Hopkins (1943). A small seta, described as between the dorsal and lateral spines of the cephalothorax (Hopkins, 1943) was not found. The abdomen has five segments plus a telson. As shown in Figure 1, A, B, the eyes are not stalked. The conical antennule (Fig. 1, C) bears a total of 5 terminal processes, the three aesthetes being longer and flatter than the two small setae. The protopodite of the antenna (Fig. 1, D) is elongated, bears two rows of minute spines on the distal half, and the small exopodite terminates in two unequal setae. The mandibles are small, with a broad cutting surface (Fig. 1, E). The endopodite of the maxillule (Fig. 1, F) bears four terminal spines, equal in length, and two slightly subterminal spines. The basal and coxal endites of the protopodite have 5 and 6 spines, respectively, and show slight bifurcation. The unsegmented endopodite of the maxilla (Fig. 1, G) also bears four terminal spines and two subterminal spines. The basal endite of the protopodite bears four spines on each bifurcation and three spines project from each lobe of the coxal endite. The scaphognathite has three setae on the outer margin of the distal portion plus two apical setae at the proximal tip. The first maxilliped (Fig. 1, H) has 4 natatory setae (cut short in the figures) on the exopodite and a spine arrangement of 2, 2, 0, 2, 5 on the 5 segments of the endopodite. The second maxilliped also has 4 swimming hairs and a 1, 1, 4 spine arrangement on the three segments of the endopodite (Fig. 1, I).

The second segment of the abdomen bears a short lateral knob and the third segment has a short hook on each side. Segments 3 to 5 also have prominent lateral spines which project caudally, overlapping the adjacent segment. A pair of small setae project dorsally from all abdominal segments other than the first. Each furcus of the telson bears a small dorsal spine and a larger lateral spine (Fig. 1, A, B). The inner margin of each furcus has three spines.

The pattern of the chromatophores was consistent for all zoeal stages. The location of those evident in Bouins-fixed larvae were: between the eyes; posterior to the eye and dorso-lateral to anterior part of gut; dorsal to gut in posterior region of cephalothorax; below base of carapace spine; mandible; distal region of basopodite of first maxilliped; middle of first abdominal segment, dorsal to gut; margin of third through last abdominal segments.

Second zoea: Eyes stalked. Number of aesthetes of antennule identical to first stage. Endopodite of maxillule bears 4 terminal and 2 subterminal spines (Fig. 2, F); basal endite bears 7 spines and coxal endite has 7 spines; a small spine is now present on outer margin of protopodite. Basal endite of maxilla (Fig. 2, G) has 8 spines and coxal endite 6 spines. Five spines are present on distal margin of scaphognathite and two project from apical tip. On third segment of endopodite of first maxilliped, one spine is added (2, 2, 1, 2, 5) (Fig. 2, H). The exopodite

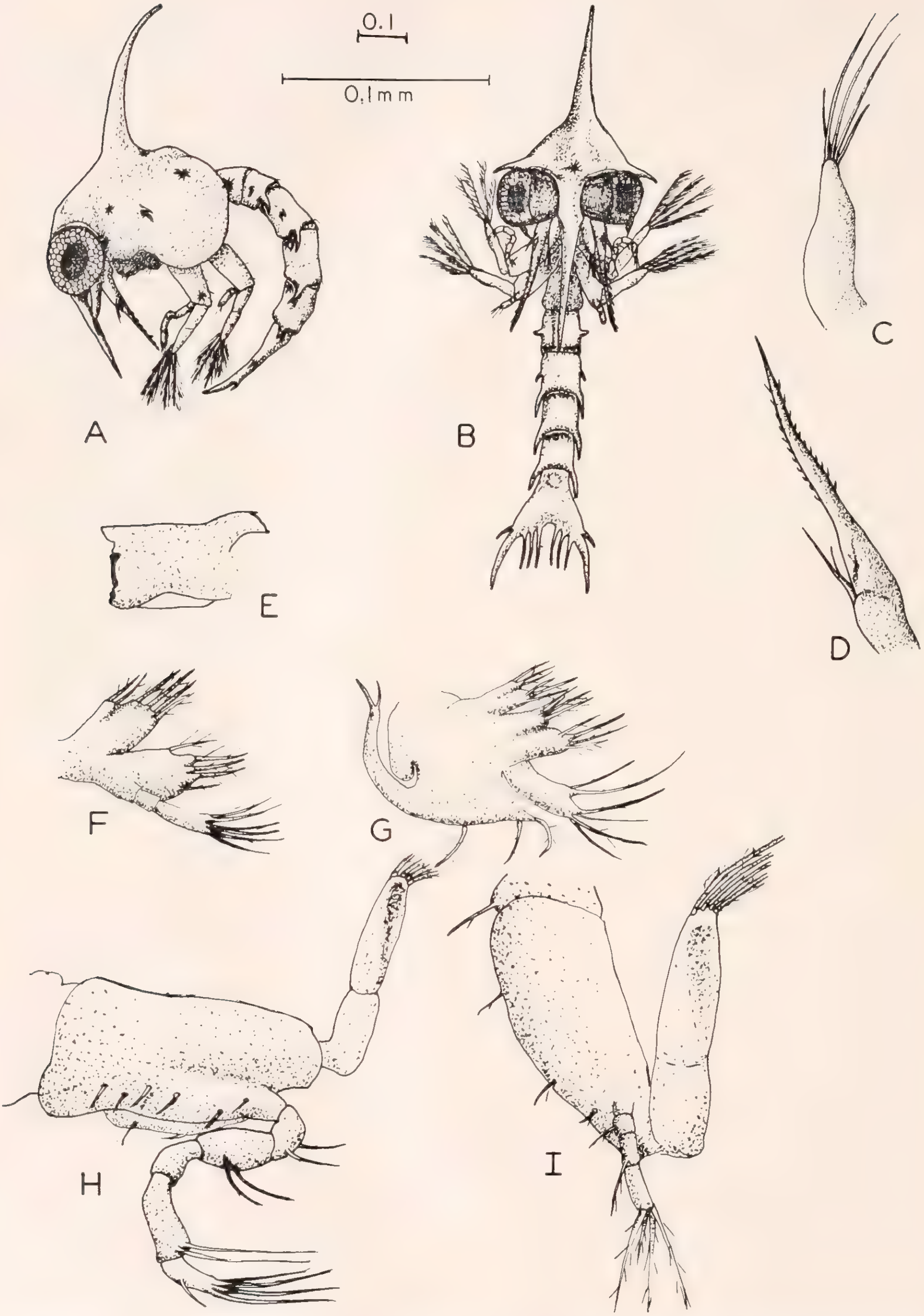

FIGURE 1. Side (A) and ventral view (B) of first zoeal stage of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, first maxilliped; I, second maxilliped. Whole zoea, × 65; appendages, × 290.

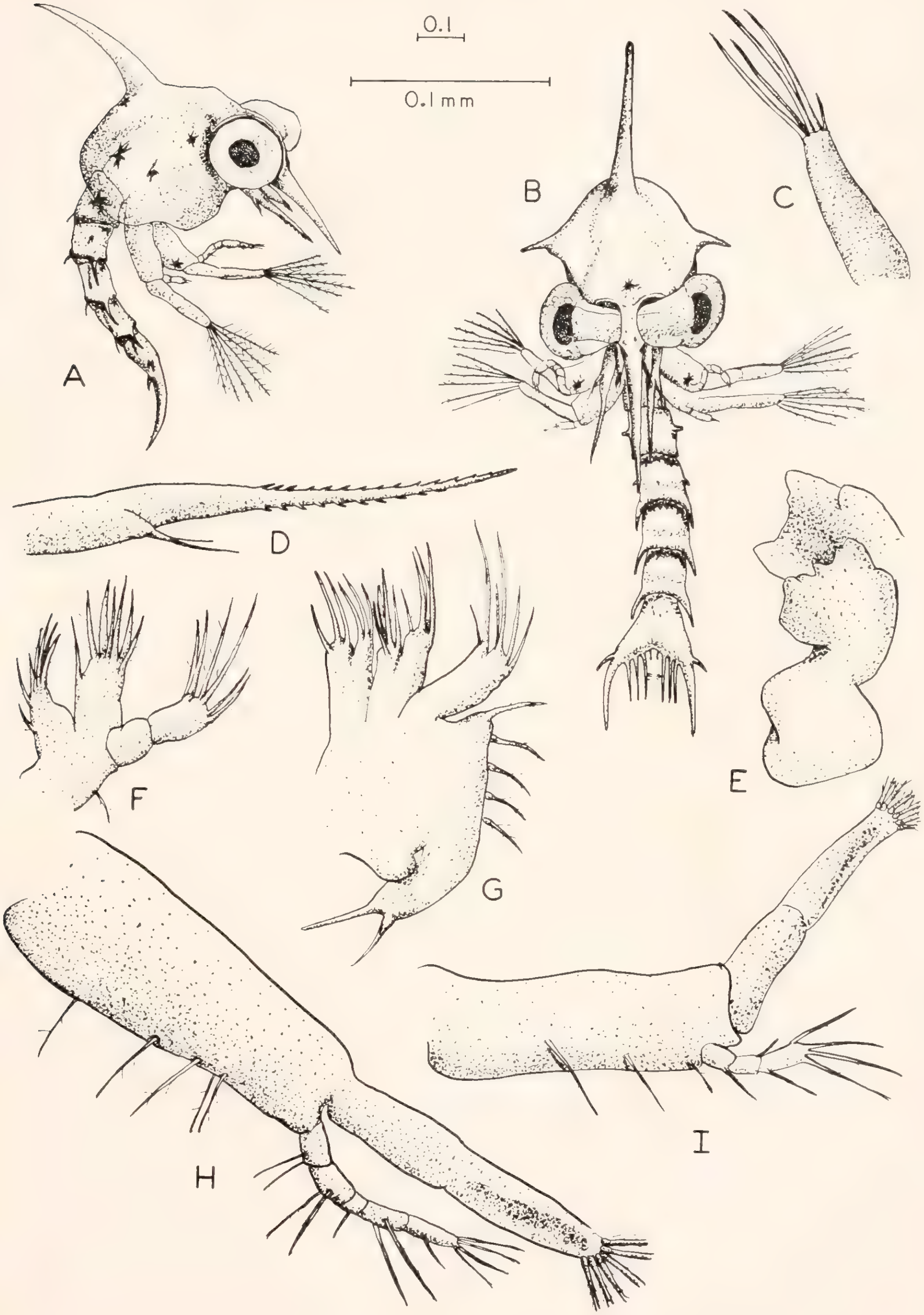

FIGURE 2. Side (A) and ventral view (B) of second zoea of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, first maxilliped; I, second maxilliped. Whole larvae, × 65; appendages, × 290.

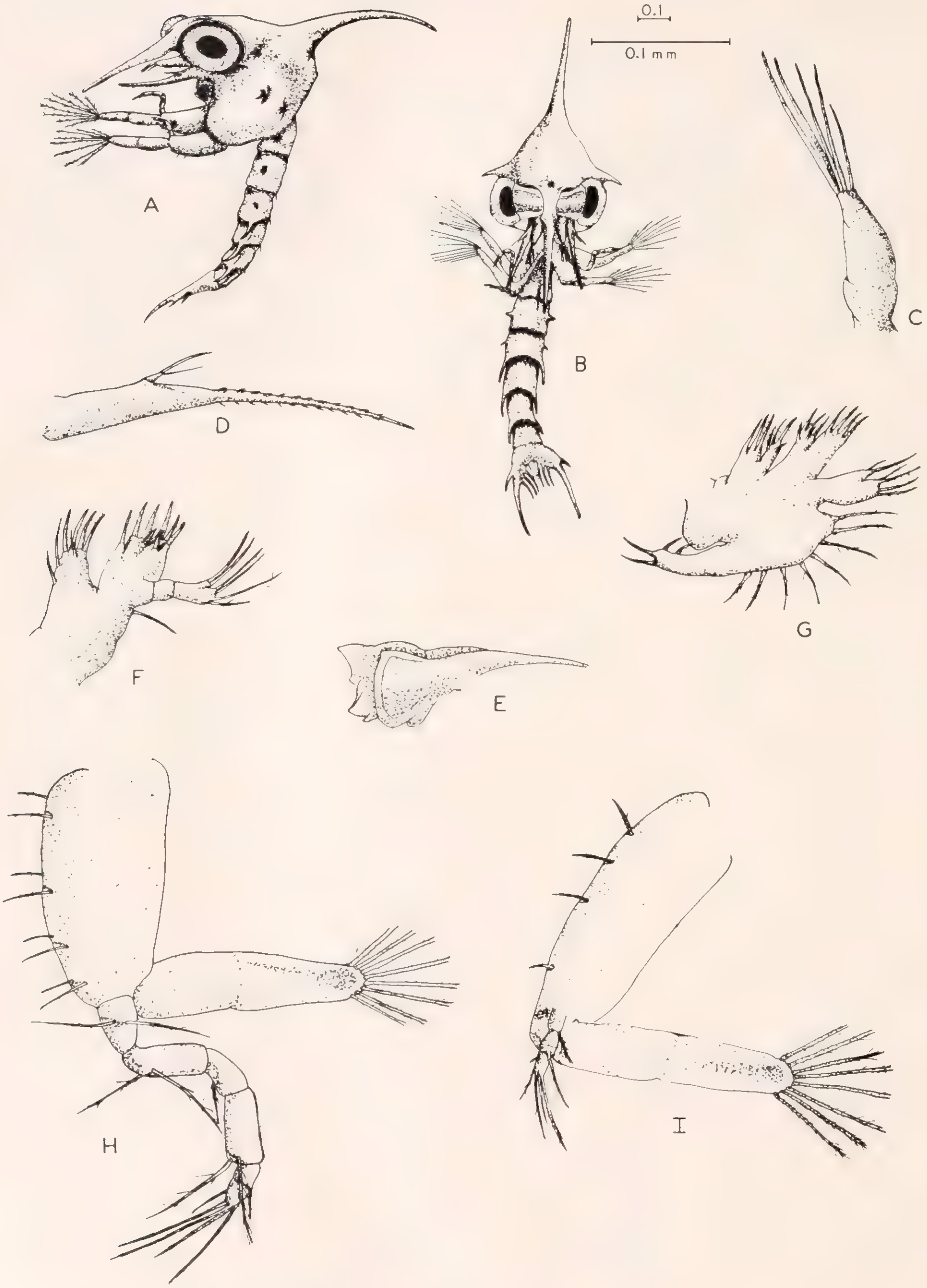

FIGURE 3. Side (A) and ventral view (B) of third zoea of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, first maxilliped; I, second maxilliped. Whole larvae, × 43; appendages, × 170.

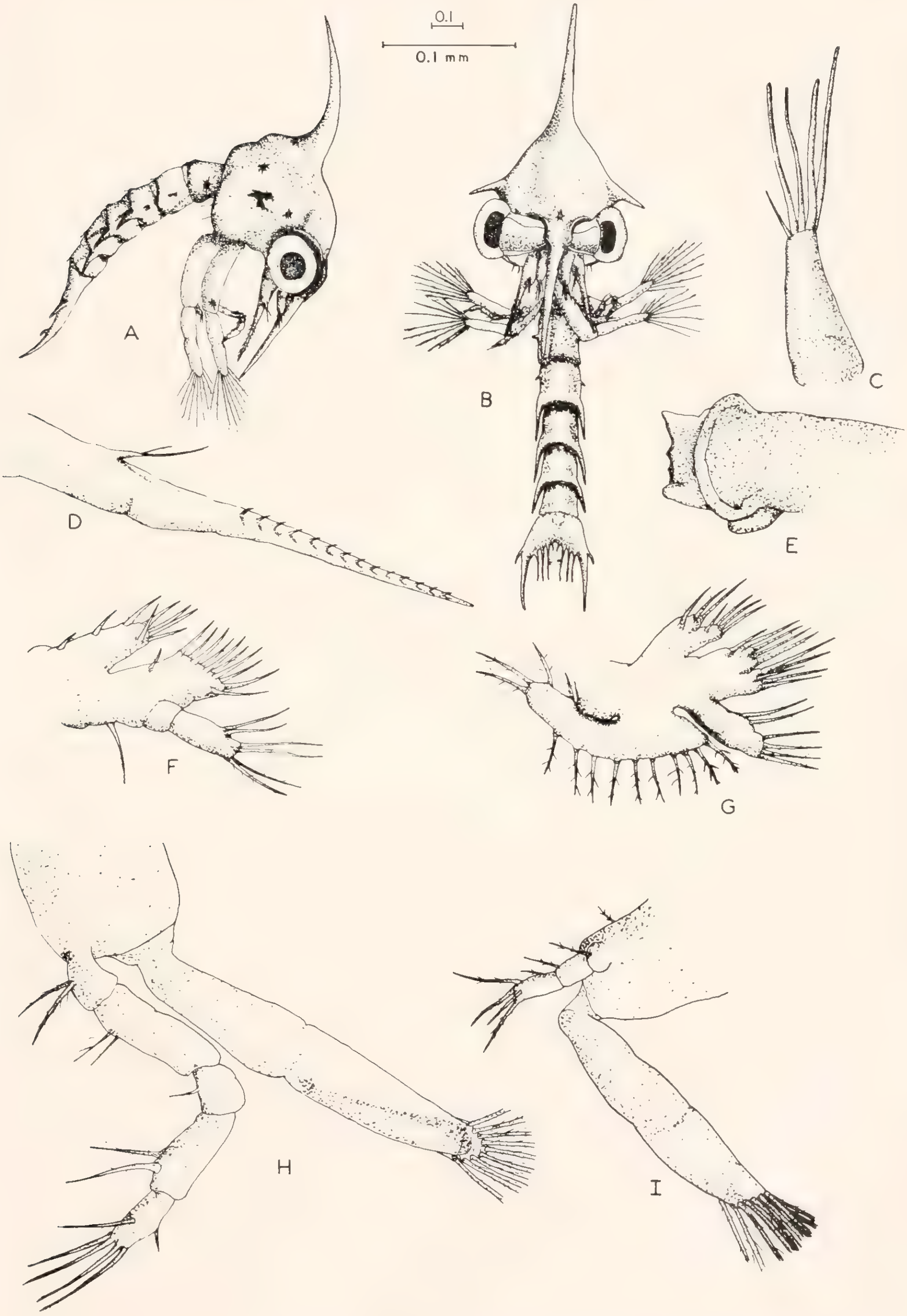

FIGURE 4. Side (A) and ventral view (B) of fourth zoea of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, first maxilliped; I, second maxilliped. Whole larva, × 43; appendages, × 170.

bears 6 plumose swimming setae. Endopodite of second maxilliped has one additional subterminal spine (Fig. 2, I). This setation, 1, 1, 5, remains constant through the remaining larval stages. Exopodite of second maxilliped bears 6 plumose swimming setae. Inner margin of each furcus of telson now bears one additional spine without setules (Fig. 2, B).

Third zoea: Setation of antennule and antenna unchanged from previous stage. The mandible (Fig. 3, E) has several small teeth in addition to the broad cutting surface. Basal endite of maxillule bears 8 spines and 7 spines project from coxal endite (Fig. 3, F). Basal and coxal endites of maxilla (Fig. 3, G) have 9 and 7 spines, respectively. Scaphognathite has 8 hairs on distal margin and 4 hairs at apical tip. A second, subterminal spine added to the fifth segment of the endopodite of the first maxilliped gives a spine arrangement (2, 2, 1, 2, 6) which remains constant in the remaining larval stages (Fig. 3, H). The exopodites of both maxillipeds terminate in 8 swimming setae (Fig. 3, H, I). A sixth segment has been added to the abdomen. It bears the small dorsal setae but does not have lateral spines (Fig. 3, B).

Fourth zoea: A slight swelling in the basal region of the antenna indicates the beginning of the endopodite bud (Fig. 4, D). A small, unsegmented palp appears with the mandible (Fig. 4, E). The basal endite of the maxillule bears 10 terminal spines and one smaller subterminal spine (Fig. 4, F). Six spines project from the terminal portion of the coxal endite and two more appear at the margin. The basal endite of the maxilla bears 10 spines and 7 project terminally from the coxal endite (Fig. 4, G). The exopodites of both the first and the second maxillipeds bear 9 swimming setae of unequal length (Fig. 4, H, I). The lateral edges of the cephalothorax have three small setae (Fig. 4, A).

Fifth zoea: The developing endopodite bud of the antenna (Fig. 5, D) is larger than in the previous stage. The maxillule remains as in the previous stage but setation of the maxilla is increased to 8 spines on the coxal endite (Fig. 5, F) and the soft hairs on the scaphognathite are increased to 20. The number of swimming setae on the first maxilliped remains as in the previous stage (9) while the second maxilliped now bears a total of 11 setae. Buds of the third maxilliped, chela, and pereiopods are visible beneath the carapace. The number of setae projecting from the edge of the carapace has increased.

Sixth zoea: A fourth aesthete, subterminal to the original 3 aesthetes and 2 setae, is added to the antennule (Fig. 6, C). Hairs appear on the small, unsegmented palp of the mandible (Fig. 6, E). A plumose spine is added to the basal segment of the endopodite of the maxillule (Fig. 6, F) and the coxal endite bears a total of 9 spines. Spines on the basal endite of the maxilla (Fig. 6, G) have increased to 13 and the marginal hairs of the scaphognathite total approximately 25. There are 11 swimming setae on the first maxilliped and 12 on the second maxilliped.

Pleopod buds appear for the first time on the abdominal segments 2 through 6 (Fig. 6, A, B). A small, non-plumose spine is added to the 8 spines within the inner margin of the telson. The number of setae on the margin of the carapace is also increased.

Seventh zoea: The terminal aesthetes of the antennule increase to 7 and 5 subterminal aesthetes have been added (Fig. 7, C). The basal portion of the antennule is swollen and there is a slight indentation in the distal half. The devel-

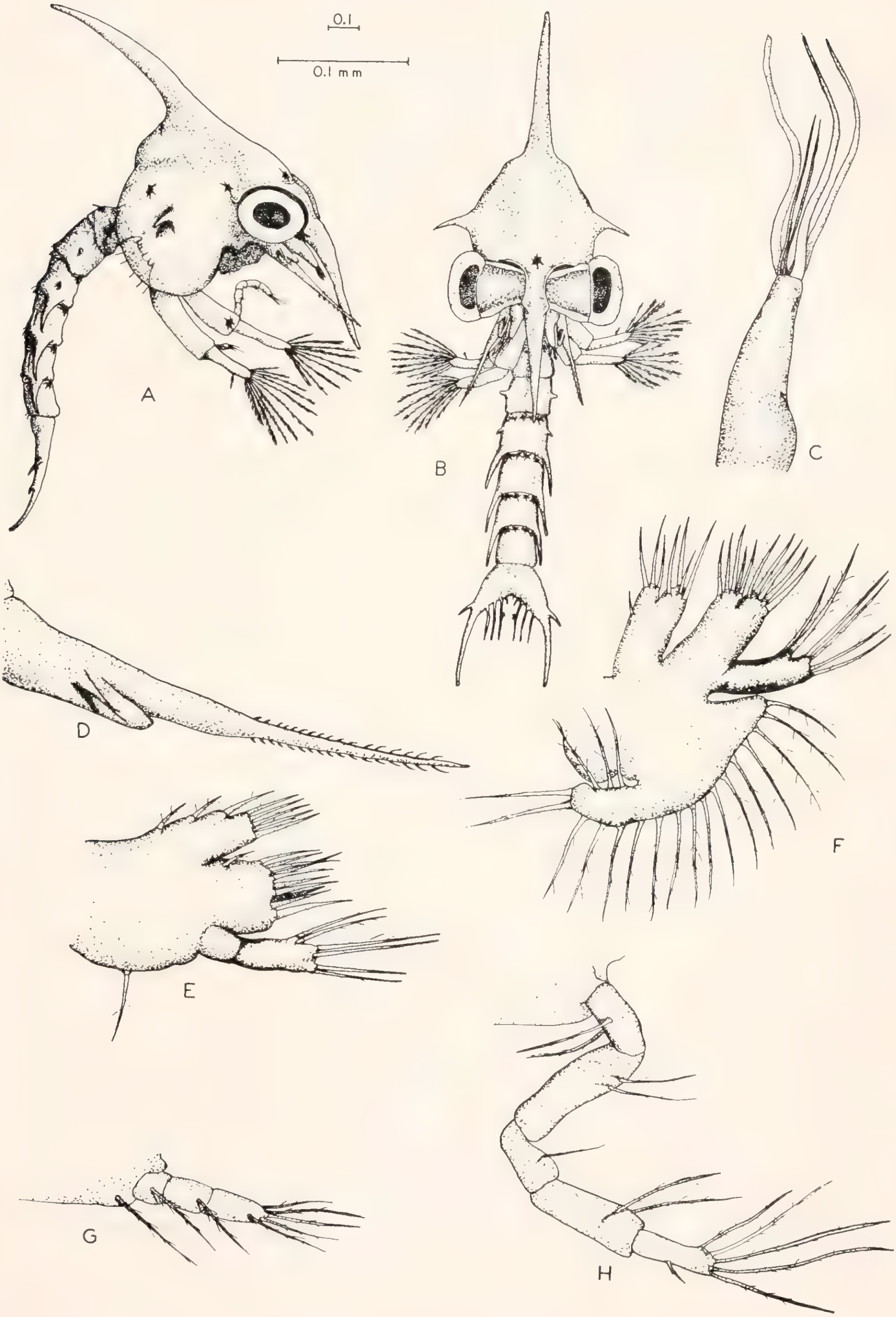

FIGURE 5. Side (A) and ventral view (B) of fifth zoea of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, maxillule; F, maxilla; G, endopodite of first maxilliped; H, endopodite of second maxilliped. Whole larva, × 43; appendages, × 170.

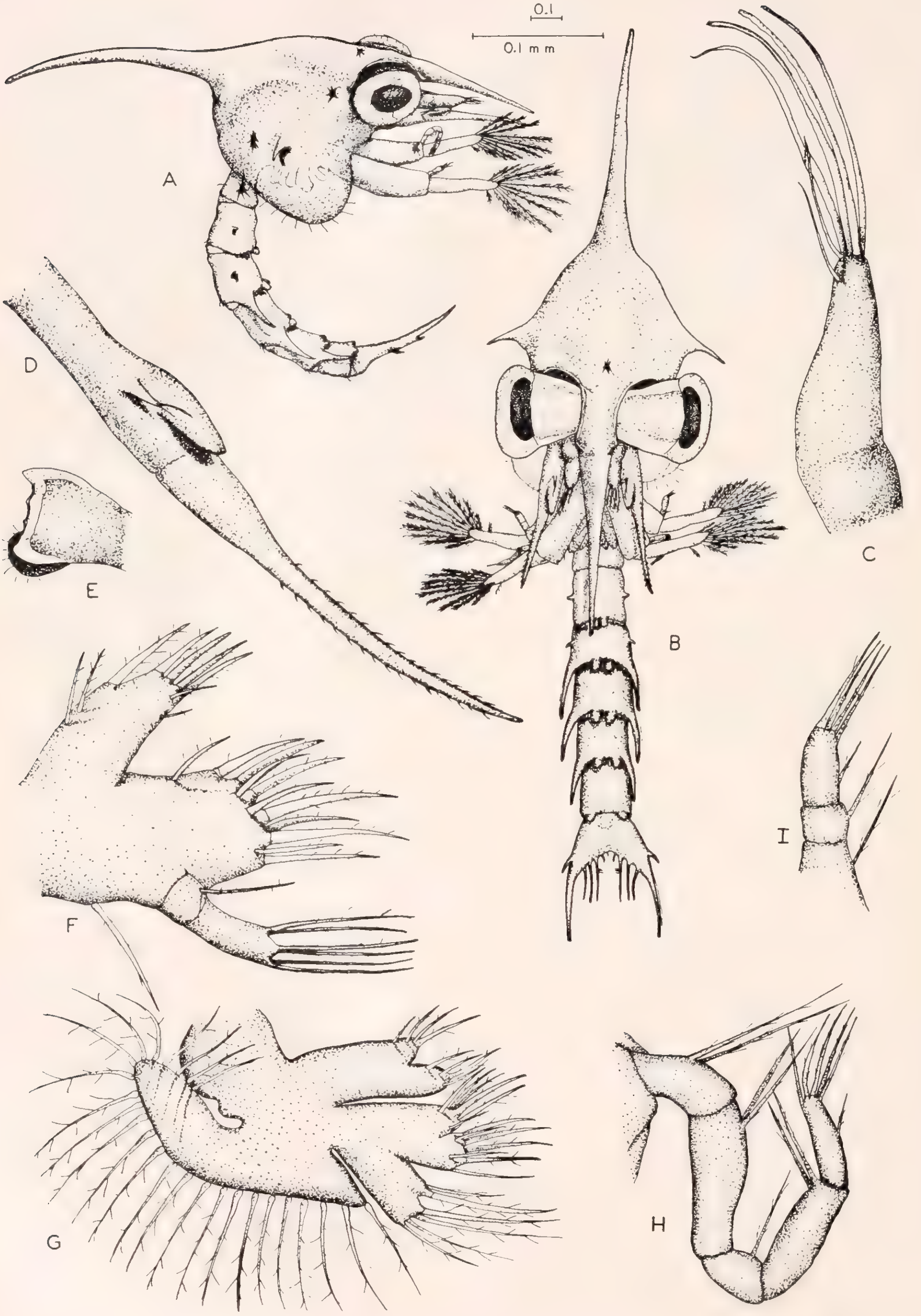

FIGURE 6. Side (A) and ventral view (B) of sixth zoea of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, endopodite of first maxilliped; I, endopodite of second maxilliped. Whole larva, × 43; appendages, × 170.

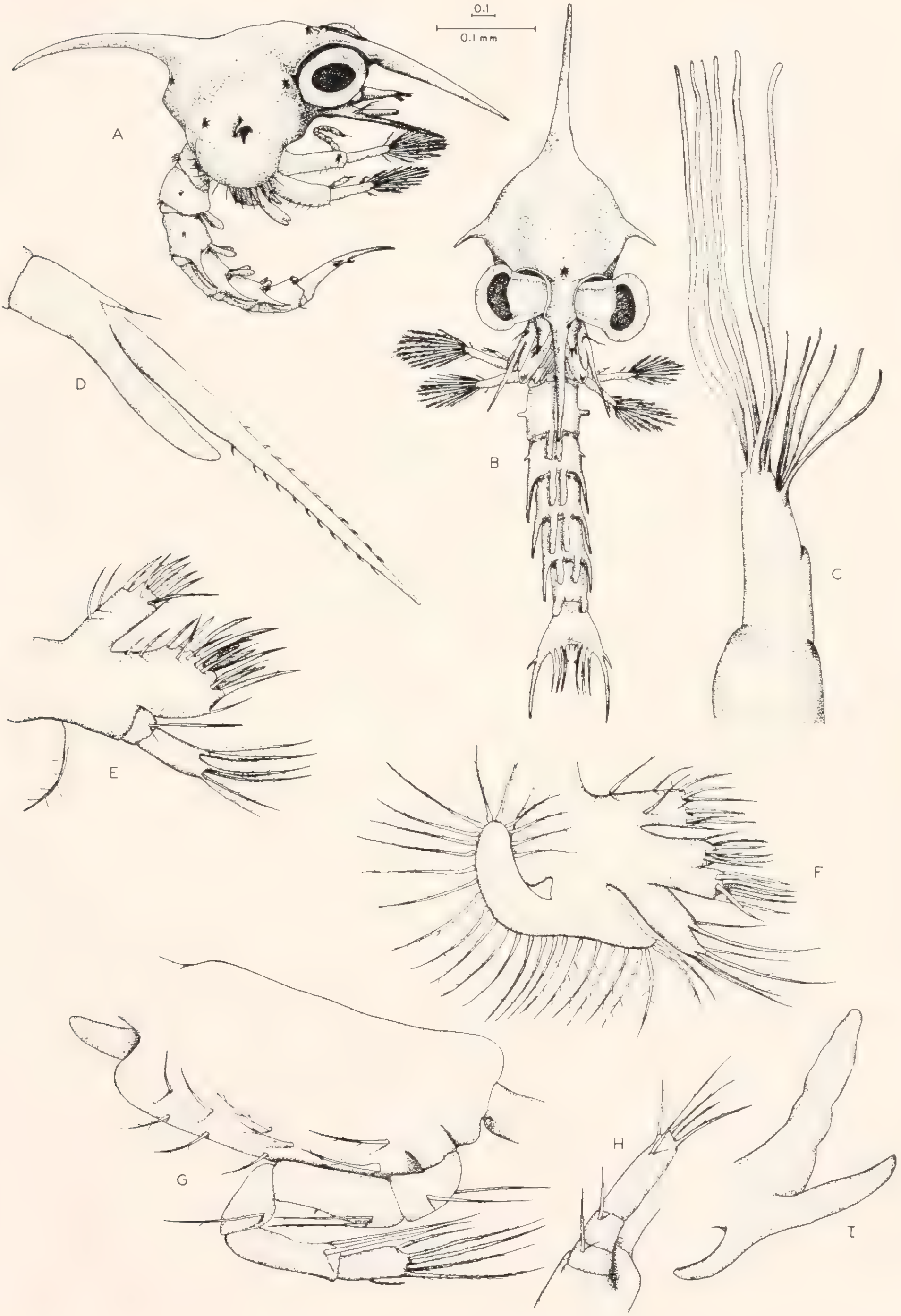

FIGURE 7. Side (A) and ventral view (B) of seventh zoea of *Callinectes sapidus* with appendages. C, antennule; D, antenna; E, maxillule; F, maxilla; G, endopodite of first maxilliped; H, endopodite of second maxilliped; I, third maxilliped. Whole larva, × 43; appendages, × 170.

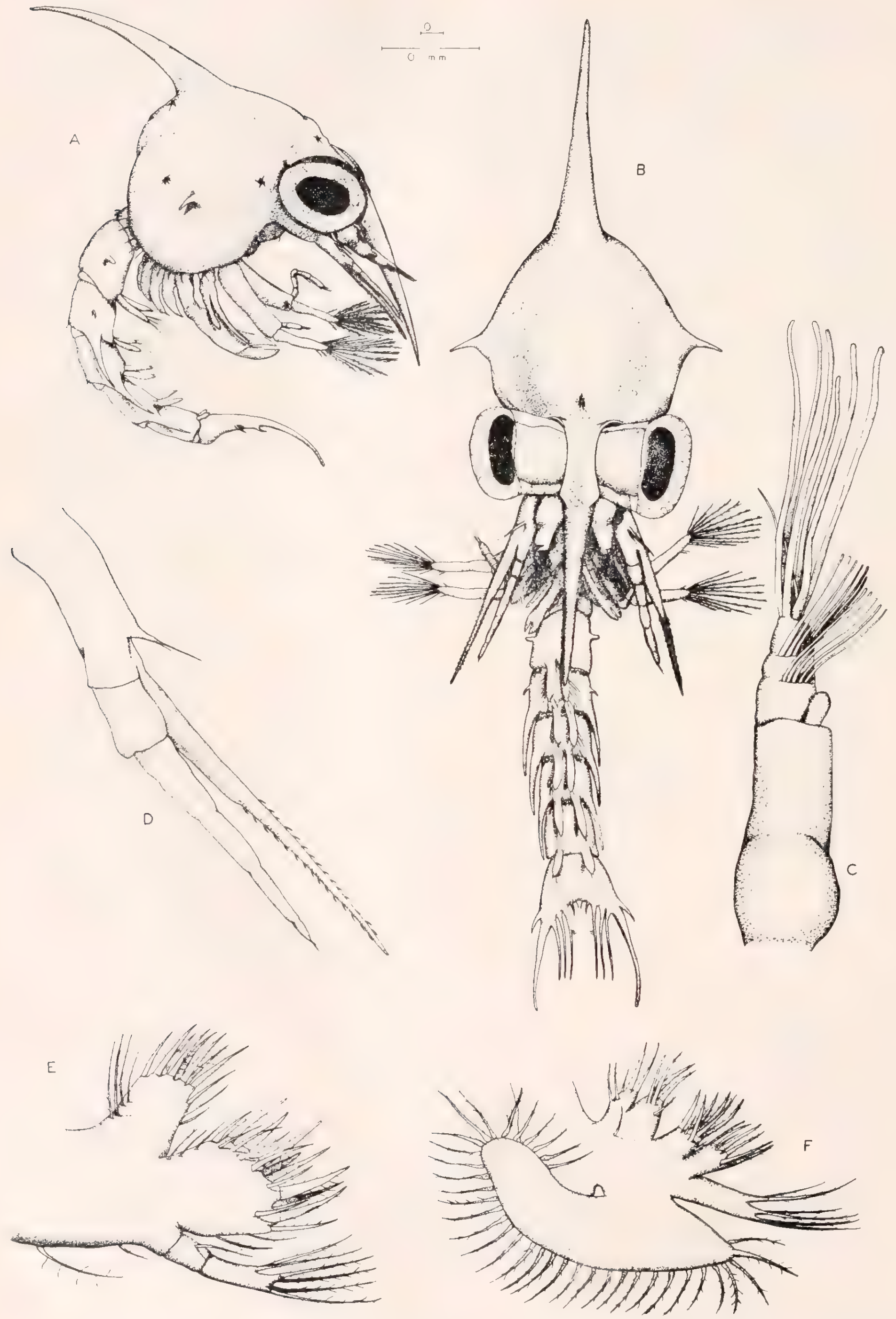

FIGURE 8. Side (A) and ventral view (B) of eighth zoea of *Callinectes sapidus* and appendages. C, antennule; D, antenna; E, maxillule; F, maxilla. Whole larva, × 32; appendages, × 135.

oping endopodite bud of the antenna (Fig. 7, D) is approximately half the length of the antenna. The basal endite of the maxillule (Fig. 7, E) bears 17 spines and the coxal endite retains the 9 spines observed in the previous stage. The spines of the basal endite of the maxilla number 14 and 10 spines are present on the coxal endite (Fig. 7, F). On the scaphognathite approximately 29 soft, plumose hairs fringe the outer margin. The swimming setae have increased to 14 on the first maxilliped and to 13 on the second maxilliped (Fig. 7, A, B). The developing thoracic appendages have increased in size and project below the margin of the carapace.

Eighth zoea: The aesthetes of the antennule are arranged in three tiers: 7 terminal, 6 subterminal, and 5 in the most basal row (Fig. 8, C). Basal portion of the antennule is more inflated and the endopodite is visible as a small knob. Endopodite of antenna (Fig. 8, D) is now almost equal in length to protopodite and shows evidence of segmentation. Basal endite of maxillule (Fig. 8, E) bears 21 spines and coxal endite has 15 spines. A second spine is added below the endopodite. Spines of the basal and coxal endites of the maxilla have increased to 15 and 10, respectively (Fig. 8, F). On the scaphognathite the plumose hairs have increased to approximately 36. Swimming setae on the first maxilliped have decreased to 12 and 14 setae are found on the second maxilliped (Fig. 8, A, B). On the first maxilliped an epipodite, partially developed, bears short setae and soft, non-plumose hairs (Fig. 9, A). Exopodite of the third maxilliped (Fig. 9, C) bears two short terminal spines and the epipodite terminates in one small, non-plumose spine. Chela and pereiopods are larger and project well beyond border of the carapace. Pleopod buds (Fig. 8, A, B) bear short non-plumose hairs. Spines on inner margin of telson total 10. Four small hairs project dorsally from posterior margin of first abdominal segment.

Megalops: Rostrum pointed, longer than antennules but shorter than antennae; eyes stalked (Fig. 9, D, E). Appendages, eyes, and margins of carapace provided with small hairs.

Antennule (Fig. 10, A) now divided into peduncle of three segments and two flagella. The unsegmented flagellum bears 6 non-plumose setae and the four segments of the other flagellum bear numerous aesthetes. The longer, terminal segment also bears two non-plumose setae. The antenna is composed of 11 segments, some of which bear setae as shown in Figure 10, B. The mandible (Fig. 10, C) has a palp of two segments with 11 bristles on distal segment. Endopodite of maxillule (Fig. 10, D) has 4 spines on terminal segment and 6 spines on first segment. The number of spines on the coxal and basal endites has increased to 17 and 25, respectively. Endopodite of maxilla (Fig. 10, E) reduced in size and bearing only three spines. There is an increase in the number of spines on endites of the protopodite and on the scaphognathite.

First maxilliped (Fig. 11, A) is considerably modified from swimming appendage of zoeal stages. Endopodite broader with 8 non-plumose setae on distal border. Exopodite of two segments, with 6 terminal setae on second segment. Epipodite well developed and fringed with long, non-plumose hairs. Second maxilliped (Fig. 11, B) has endopodite of 4 segments with stout spines on terminal segment. Exopodite is two-segmented with 6 terminal hairs. The epipodite is small. Third maxilliped (Fig. 11, C) with large endopodite bearing numerous spines on

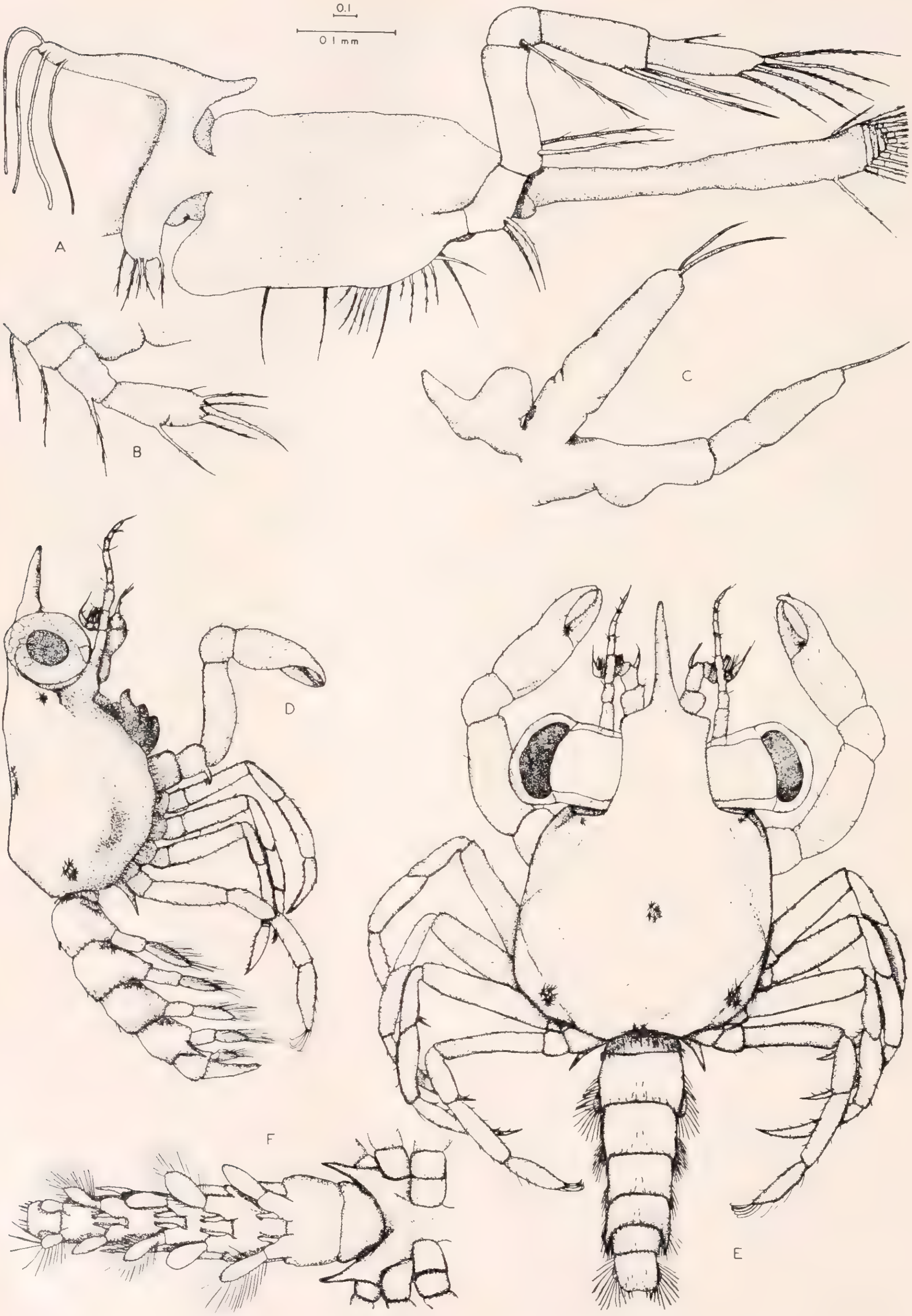

FIGURE 9. Appendages of eighth zoea and side and dorsal view of megalops of *Callinectes sapidus*. A, first maxilliped; B, second maxilliped; C, third maxilliped; D, side view of megalops; E, dorsal view of megalops; F, ventral view of abdominal segments of megalops (setae removed on alternate pleopods for clarity). Whole megalops, × *32*; appendages, × 135.

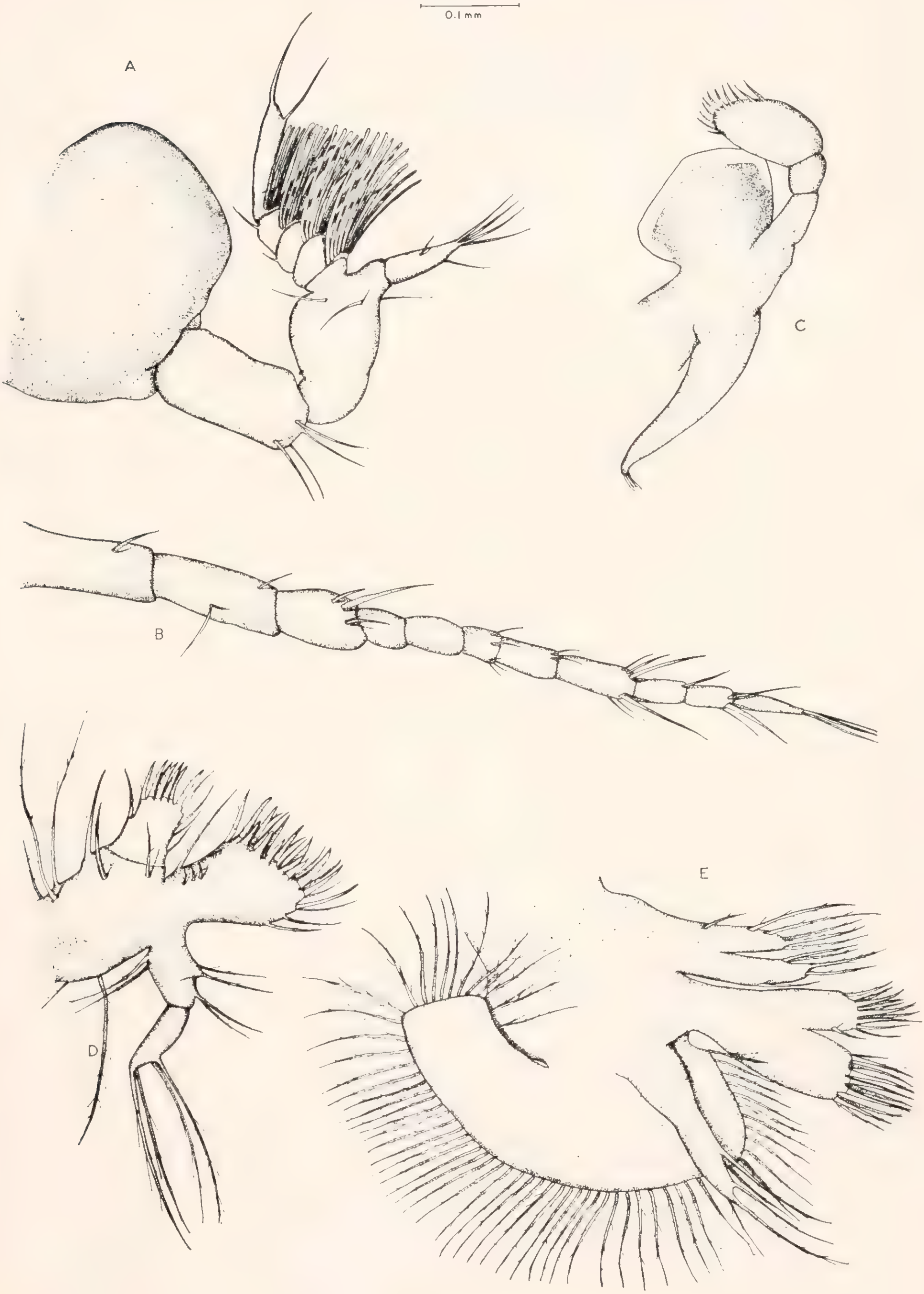

FIGURE 10. Appendages of megalops of *Callinectes sapidus.* A, antennule; B, antenna; C, mandible; D, maxillule; E, maxilla. × 135.

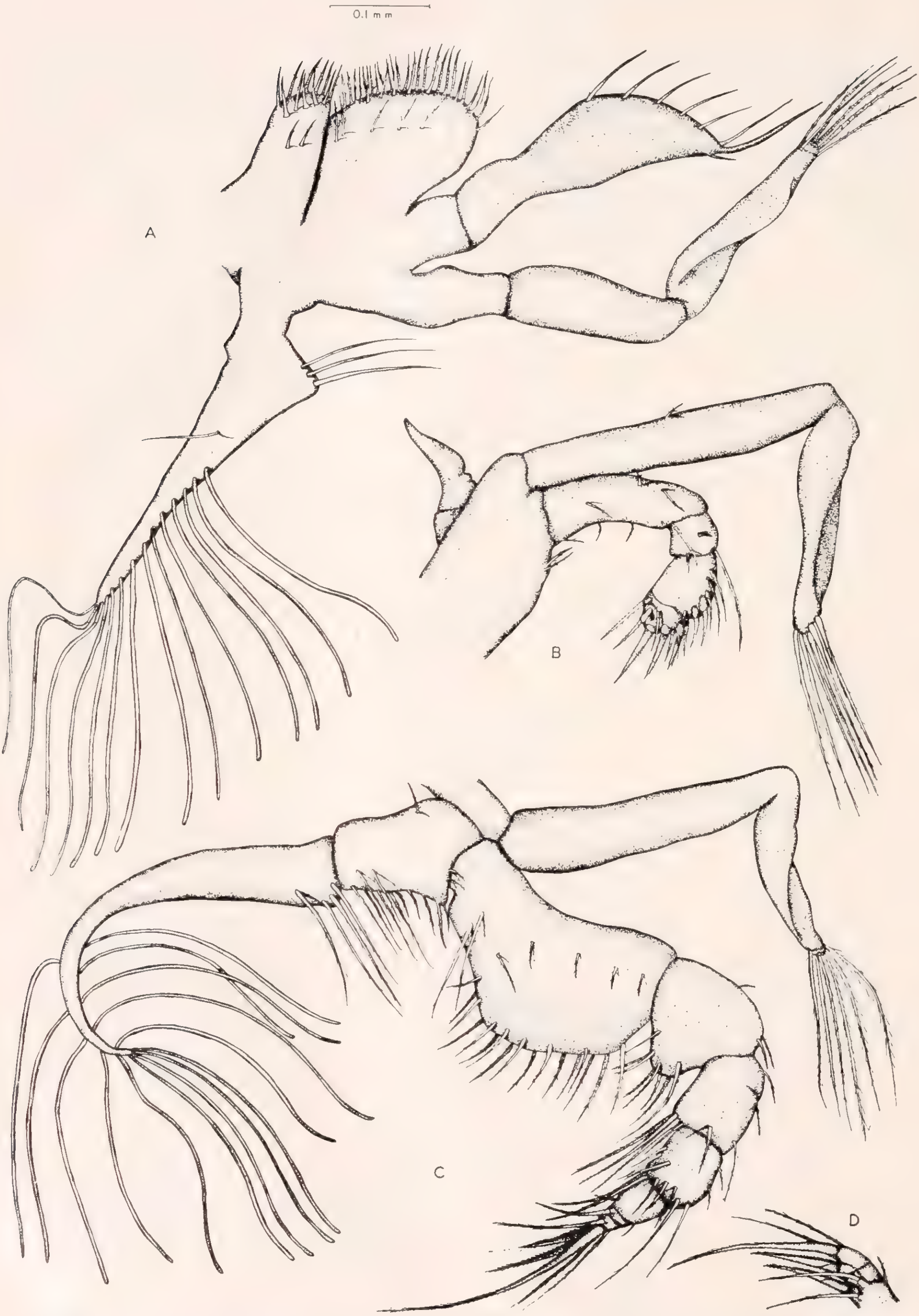

FIGURE 11. Appendages of megalops of *Callinectes sapidus.* A, first maxilliped; B, second maxilliped; C, third maxilliped; D, terminal segment of third maxilliped. × 135.

all segments; exopodite unsegmented and bearing 6 terminal setae; epipodite fringed at distal portion by soft, non-plumose hairs. Spine on lateral surface of basi-ischiopodite of cheliped (Fig. 9, D, E), and dactylopodite of fifth pereiopod has 5 terminal spines. Cornua project from posterior edge of cephalothorax (Fig. 9, E, F). Fifth abdominal segment retains lateral spines, projecting caudally past the smaller sixth abdominal segment (Fig. 9, D, F). Endopodites developed on all pleopods other than fifth pair. Exopodites of pleopods on segments 2 through 6 with 24, 23, 22, 21, and 12 long, non-plumose setae (Fig. 9, F). Four small, curled spines are found on inner surface of endopodite of the pleopod of

Table II

Time of molting, expressed as days after hatching, for larvae of C. sapidus in salinity-temperature combinations in which development was complete or partially complete

	° C. \ p.p.t.	20.1	26.7	31.1
Molt I	25	6–13	a) 7–9 c) 6–12 d) 7–9	7–13
	30		5–11	
Molt II	25	12–16	a) 10–12 c) 10–20 d) 10–12	11–19
	30		11–16	
Molt III	25	17–27	a) 15 c) 17–26 d) 14–23	15–27
	30		14–18	
Molt IV	25	24–30	a) 19 c) 20–32 d) 18–26	20–29
Molt V	25	28–34	a) 22 c) 24–39 d) 22–33	24–39
Molt VI	25	38	a) 27 c) 28–39 d) 26–38	29–43
Molt VII (to megalops)	25	43	a) 31 c) 35–49 d) 32–45	35–47
Molt VIII (to crab)	25	50	a) 37 c) 50–55 d) 39–53	45–55

the second abdominal segment and three similar spines are present on endopodites of remaining pleopods. Telson with 6 to 8 short spines on posterior border.

Larval development

Hatching was observed at all experimental salinities except 15 p.p.t. In water of 20.1 p.p.t.–32 p.p.t. the zoeae hatched as first stage larvae and the so-called "pre-zoea" was never observed. Complete development to the first crab stage occurred in the four temperature-salinity combinations shown in Table I.

As shown in Table II, the time of molting of the three series of larvae maintained at 26.7 p.p.t., 25° C. (Series a, c and d) was similar. The first molt occurred within the same period of time for larvae at 20.1, 26.7, and 31.1 p.p.t. At these three salinities there was also little difference in the time of the later molts (Table II) and in the range of time for complete larval development (Table III). The only difference in time required for total development was found in the series of larvae hatched and reared at 32 p.p.t. After dilution to 28 p.p.t. on day 41, at which time all the larvae had been either sixth or seventh stage zoea for some time, some molted to the megalops stage and eventually metamorphosed to the first crab on day 61.

TABLE III

Number of days observed for development of all zoeal stages (Z), duration of the megalops stage (M), and time for total development to the first crab stage (T) for larvae of Callinectes sapidus hatched and maintained at 25° C. in the salinities shown

20.1			26.7			31.1			32.0*		
Z	M	T	Z	M	T	Z	M	T	Z	M	T
43	7	50	a) 31	6	37	35–47	10–20	45–57	46	15	61
			b) 35–49	7–9	44–56						
			d) 32–45	6–9	38–53						

* Diluted to 28 p.p.t. on day 41.

The one series in which zoeae completed the first three molts at 30° C., 26.7 p.p.t., shows no significant difference in the time of the molts in spite of the additional 5° C. in temperature (Table II).

Mortality of *C. sapidus* larvae (Table IV) was highest during the first two zoeal stages in all temperature-salinity combinations. In all salinities larvae never went beyond the first zoeal stage when maintained at 20° C. At 10.5 and 15.6 p.p.t. mortality was also highest during the first stage at all three temperatures. Larvae maintained at one temperature-salinity combination, 25° C., 15.6 p.p.t., did molt to the second stage but died within a few days (Table IV). Once the second molt had been completed some of the remaining larvae usually lived to complete metamorphosis to the crab.

The number of zoeal stages of *C. sapidus* varied from 7 to 8. Most of the larvae which molted to the megalops did so following the seventh zoeal stage but one completed 8 zoeal stages and then metamorphosed to the megalops. The majority of the eighth stage zoeae died without additional molts. The variation

TABLE IV

Mortality of larvae of Callinectes sapidus at different stages, expressed as per cent of original number of zoeae, in those temperature-salinity combinations which permitted at least partial development.

	p.p.t. ° C.	15.6	20.1	26.7	31.1
Stage I	25	95	42	a) 72.2 c) 30.0 d) 11.0	53.3
	30		95	58.3	60.1
Stage II	25	5	36	a) 16.7 c) 57.5 d) 42	22.8
	30		5	37.0	37.0
Stage III	25		11	a) 5.5 c) 3.5 d) 10.0	12.0
	30			2.7	2.8
Stage IV	25		8	a) 0.0 c) 2.0 d) 5.0	0.6
	30			1.8	
Stage V	25		1	a) 0.0 c) 0.0 d) 9.0	4.6
Stage VI	25		1	a) 0.0 c) 0.0 d) 9.0	0.6
Stage VII	25		0.0	a) 0.0 c) 0.0 d) 4.0	4.0
Megalops	25		0.0	a) 0.0 c) 4.3 d) 1.0	0.0

in number of stages occurred within one salinity-temperature combination (26.7 p.p.t., 25° C.) as well as in the other salinities. The megalops stage metamorphosed directly to the first crab stage.

DISCUSSION

Larval stages

The only existing description of all larval stages of *Callinectes sapidus* (Churchill, 1942) is based entirely on reconstruction from planktonic material. Hopkins

(1943, 1944) was able to rear *C. sapidus* through the first three zoeal stages and concluded that Churchill's (1942) description of the larvae included zoeae from several species. Reconstruction of the stages in larval development is always susceptible to this error in an area which includes more than one species. By rearing zoeae, liberated in the laboratory from the egg mass of an identified female, the species can definitely be known and confusion resulting from the mixing of larvae from several species is avoided.

The larval development of many crabs has been reported to include a "pre-zoeal" stage. The "pre-zoea" is described for *C. sapidus* by Robertson (1938) and by Churchill (1942). In the present study the larvae, although varying considerably in size, always hatched as first zoeae in salinities of 20.1, 26.7, 31.1 and 32 p.p.t. Lochhead, Lochhead and Newcombe (1942) observed that 90 per cent of the eggs hatched as first zoeae under "favorable conditions" but that "pre-zoeae" were obtained if conditions were "unfavorable." Sandoz and Rogers (1944) found hatching to be associated with salinity: below 20 p.p.t. the per cent of larvae which emerged as "pre-zoeae" increased.

The setation of the maxillipeds of *C. sapidus* larvae has been given by Churchill (1942) and, for the first three stages reared in the laboratory, by Hopkins (1943, 1944). The results of the present study agree with previous findings for the first two zoeal stages. Beginning with the third zoea, however, our description does not agree with that given by previous workers. Churchill (1942) gives 6 and 7 setae for the first and second maxillipeds, respectively, Hopkins (1944) found 8 and 9 setae, and we observed 8 swimming setae on each maxilliped. Hopkins (1944), describing a fourth stage zoea obtained from the plankton, gave the setation of the first and second maxillipeds as 8 and 10 while we found it to be 9 and 9.

Robertson (1938) and Churchill (1942) put great emphasis on the cornua as a distinguishing feature of the *C. sapidus* megalops. Aikawa (1937) described the megalops of several species of *Portunus*, obtained from the plankton, and included the cornua in the figures for these species. Aikawa (1937) also mentioned the hook on the basi-ischiopodite of the chela and the lateral spines on the fifth abdominal segment of the megalops. Lebour (1928), describing the megalops of *Portunus puber* reared from the egg in the laboratory and megalops of other species of *Portunus* obtained from the plankton, did not figure or describe these three characters for any species of *Portunus*.

The present description of setation of the maxillule and maxilla agrees with Hopkins' (1943, 1944) findings for the first three stages. In many previous studies on larvae of the Brachyura the zoeae have been staged very largely by differences in the number of swimming hairs on the first and second maxillipeds. Aikawa (1937) compares setation of the maxillule and maxilla for a great variety of brachyuran larvae but includes only the first stage zoea. In each zoeal stage of *C. sapidus* examined in the present study it was found that there was always a progressive change in the setation of the maxilla. Setation of the maxillule was also different, except for the fourth and fifth zoeae. Hence these appendages, and others, may be important in staging larvae of different crabs. The significance of these appendages as diagnostic characters, however, will have to await a comparative study of all stages in the larval development of other species of crabs.

Larval development

Although the effects of salinity and temperature on larval development of other crabs have been studied (Coffin, 1958; Costlow and Bookhout, unpublished results), Sandoz and Rogers (1944, 1948) give the only available data dealing specifically with the blue crab, *Callinectes sapidus.* In the present study on larvae of this species the results agree closely with those reported for the first zoeal stage by Sandoz and Rogers (1944).

If the salinity were reduced beyond 20.1 p.p.t. by dilution with distilled water, the zoeae did not usually live beyond the first molt. Sandoz and Rogers (1944) obtained some second zoeae at 20 p.p.t. and 25 p.p.t. (24°–29° C.) but the few which molted to the third stage did not live. In the present study the time of molting (Table II) was quite variable, even within one salinity-temperature combination. Sandoz and Rogers (1944) reported an average of from 6–7 days for the first molt at 20 and 25 p.p.t., 24°–29° C., although some larvae molted as late as the eleventh day. In the present study the first molt was completed in from 5 to 13 days in several salinity-temperature combinations (Table II). The later molts became more variable in time in all three salinities in which development was complete.

In the present study isolated larvae did molt and successfully complete development to the crab stage. Sandoz and Rogers (1944) did not observe any molting among isolated larvae and all eventually died.

One series of larvae, hatched and maintained for 41 days at 32 p.p.t., was of particular interest. The sixth and seventh stage zoeae were active but did not molt to the megalops. On day 41 the larvae were divided into three groups. The water containing one group of zoeae was reduced from 32 p.p.t. to 28 p.p.t. in approximately 4 hours. All zoeae of this group died within 24 hours. Water containing the second group of larvae was diluted to 28 p.p.t. over a period of approximately 24 hours. Five days later one zoea molted to the megalops and on day 61, metamorphosed to the crab. Larvae of the third group, retained at 32 p.p.t., died without any additional molting. While the number of larvae used should not be relied upon for any definite conclusions, it may be pointed out that the larvae hatched and reared at 31.1 p.p.t. completed metamorphosis to the crab without dilution to a lower salinity. Thus it would appear that the threshold which exists in the upper range of salinities is abrupt and well defined.

At 25° C. the duration of the megalops stage (6–9 days) was similar for larvae maintained at 20.1 and 26.7 p.p.t. (Table III). In the higher salinity (31.1 p.p.t.) 10–20 days were required and in water diluted from 32 p.p.t. to 28 p.p.t., the megalops persisted for 15 days before molting to the crab. Sandoz and Rogers (1948) found little difference in the time required for the megalops to molt to the crab in 20 p.p.t. and 31 p.p.t. The 2.6–2.9 days which they record, however, were for stages obtained from the plankton and the exact age could not be known. If, as suggested by Sandoz and Rogers (1948), the megalops were approximately 2–3 days old when first obtained, the total period of 5–6 days would correspond closely with our results at 20.1 and 26.7 p.p.t.

Churchill (1942) estimated that zoeal development of *C. sapidus* in the Chesapeake Bay was completed in approximately one month. Zoeal development in the laboratory required a minimum of 31 days and a maximum of 49 days, at various salinities. In the laboratory 7 zoeal stages and one megalops stage were observed whereas Churchill (1942) described 5 zoeal stages and one megalops from planktonic material.

The use of *Artemia* nauplii has proven successful in rearing a variety of decapod larvae (Broad, 1957; Chamberlain, 1957; Knudsen, 1958; Coffin, 1958; Costlow and Bookhout, unpublished results) and Cirripedia larvae have been reared from hatching to settling and metamorphosis on *Arbacia* eggs (Costlow and Bookhout, 1957, 1958). The combination of *Arbacia* eggs and recently hatched *Artemia* nauplii used in the present study provides a source of motile food of different sizes. In our experience with other decapod larvae, also reared at different salinity-temperature combinations, the zoeae were vigorous and fed actively. *C. sapidus* larvae, even after completion of several molts, often appeared fragile and less vigorous than larvae of other species. Algae have been used unsuccessfully in attempts to rear the larvae of many decapods by previous workers. We have found that while *C. sapidus* zoeae will ingest many of the unicellular algae and live 10–13 days, the larvae never molt. Even though the gut is full of the cells, and fecal pellets are numerous, further development does not occur. In the present study algae were not used because zoeae which were provided algae have been observed to feed less actively on *Artemia* nauplii. Dean (1958) has suggested that what have been interpreted as differences in the nutritive quality of algae may represent "resistance" to digestion.

The 7 zoeal stages described for *C. sapidus* may not represent the number of stages present in development under natural conditions. A main criticism of laboratory rearing has been that suboptimal conditions may produce "abnormal" stages and give a picture of larval development which is not consistent with that assumed to be found in the natural environment (Gurney, 1942). In the few existing examples of successful rearing of Brachyura in the laboratory no reference is made to "extra" or "abnormal" stages. Lebour (1930), dealing with larvae of the Anomura, noted that 5 larval stages usually represent the normal development of *Galathea* but that the fourth and fifth stages may be omitted. In the Macrura, Templeman (1936) found a stage in the larvae of *Homarus americanus*, intermediate in form between the recognized third and fourth stages, and attributed it to unfavorable rearing conditions. More recently, Broad (1957) has shown that the number of larval stages of *Palaemonetes* is directly associated with the availability of food. Lebour (1928), discussing the primitive nature of the Brachyrhyncha larvae, considers *Portunus* as the most primitive because of the many zoeal stages (5) and the spine structure of the telson. The 7 zoeal stages described for *C. sapidus*, a form closely allied to *Portunus*, may indicate a primitive adaptive quality which has, in part, accounted for the success of this species all along the Atlantic and Gulf coasts.

If larval development is complete, and the post-larval stage is reached, it appears erroneous to refer to "abnormal" stages of development. Our present knowledge of the factors involved in the physiology of larval development of the Brachyura is too limited to predetermine the number of larval stages required for the development of any crab.

SUMMARY AND CONCLUSIONS

The larvae of *Callinectes sapidus* Rathbun were reared in the laboratory from hatching to the post-larval stages under conditions which combined 20° C., 25° C., 30° C., and 6 salinities (10.5, 15.6, 20.1, 26.7, 31.1 and 32 p.p.t.). Of the 3,014 zoeae maintained in 15 different combinations of salinity and temperature 1–8 per cent completed development at 25° C., in salinities of 20.1, 26.7, and 31.1 p.p.t. The zoeal stages and megalops stage are described and figured. From this study the following conclusions may be made:

1. Eggs hatched as first zoeae and the "pre-zoea" stage was not observed.
2. Seven zoeal stages and one megalops stage were observed in the complete development to the first crab in the laboratory. An eighth zoeal stage was sometimes observed but usually did not complete metamorphosis to the megalops.
3. Setation of the maxillipeds and the maxillule showed a progressive increase with each larval stage and may be useful in the staging of species obtained from the plankton.
4. Development to the megalops required a minimum of 31 days and a maximum of 49 days. The megalops persisted from 6–20 days in the salinities used.
5. There is no significant difference in time of zoeal development in water with salinities of 20.1–31.1 p.p.t.
6. At a higher salinity (31.1 p.p.t.) a greater length of time is required for the megalops to complete metamorphosis to the first crab than when reared in lower salinities (20.1–26.7 p.p.t.).
7. Even though some zoeae completed development in salinities of 20.1–31.1 p.p.t. mortality was usually highest during the first two zoeal stages. Below 20.1 p.p.t. larvae rarely completed the first molt.
8. The large number of zoeal stages may not reflect development under natural conditions. The 7 zoeal stages may, however, indicate a primitive adaptive quality which has accounted for the success of *Callinectes sapidus* Rathbun along the Western Atlantic and Gulf of Mexico coasts.

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STUDIES ON THE FORM OF THE AMPHIBIAN RED BLOOD CELL

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To a student of cell form the erythrocyte is an ideal subject for investigation. It is a free cell, not permanently involved in contact with other cells, and it has a definite and relatively simple form. I recently published an account of a model which was proposed as a partial explanation for the elliptical form of the amphibian red cell (Davison, 1957). Since the model has served as a guide to the present work, I will briefly describe its salient features as an introduction to these further observations.

The blood cells of the newt *Triturus viridescens* approximate thin elliptical discs in form. Viewed as plane elliptical figures, triploid cells have approximately 1.5 times greater area than diploid blood cells, but are apparently no greater in thickness, a relationship similar to that described by Fankhauser for 2n and 3n skin epidermal cells (Fankhauser, 1952). Not only are the 3n cells larger, they clearly have a different shape than 2n cells, being more eccentric regarded as elliptical figures. Using the ratio of the major to minor axes (a/b) as an index to cell form, 2n and 3n *Triturus* red cells were found to have mean eccentricities of 1.55 and 1.82, respectively.

It has long been recognized that liquid drops can, under the proper physical conditions, simulate many protoplasmic structures (Thompson, 1942). Reasoning that the blood cell exists in a system of cylinders, the blood vessels, I thought it might prove interesting to examine the form characteristics of a fluid drop in contact with a cylindrical surface. If one places a large (29 cm. in diameter) cylindrical glass vessel with the axis horizontal, and pours mercury on the inside of the cylinder, the mercury will assume the form of a flat elliptical disc. Adding more mercury to the pool increases both the area and the eccentricity of the drop but does not appreciably increase its thickness. The model thus simulates the form differences observed between 2n and 3n blood cells. In the model the mercury is in contact with the cylindrical surface through the deforming force of gravity. In the animal it is clear that the blood cells are applied to the wall of the capillary but are not so oriented during their passage through larger vessels. No significant differences were found in the diameter of 2n and 3n capillaries, an essential point, since it is also clear from the model that the larger the cylinder the less eccentric the fluid drop. The latter observations from the model suggest that changes in capillary diameter should lead to alterations in red cell form, with an increase in

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[1] I would like to express my sincere appreciation to Dr. Gerhard Fankhauser of Princeton University who so generously offered me the use of his laboratory and supported this work through a grant from the Pfeiffer Foundation.

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cell eccentricity following a decrease in capillary diameter and a decrease in cell eccentricity following an increase in capillary diameter.

With this background in mind, the further objectives of the study may be stated as follows:

(1) To examine cell form when expressed as a continuous function of cell area, especially with reference to the cross-sectional area of the capillary.

(2) To examine the effect of changes in capillary diameter on red cell form under conditions of constant cell area.

(3) To quantitatively relate these variables.

ANIMALS AND METHODS

Since both diploid and triploid spanish newts (*Pleurodeles waltlii*) were available, this animal was selected to examine cell eccentricity as a function of cell area. *Pleurodeles* cells are less eccentric than those of *Triturus,* better permitting an analysis of the manner in which the blood cell approaches the circular form. The studies on adult *Triturus* followed the accidental discovery that cold-adapted (8.5° C.) animals have much more eccentric blood cells than the same animals maintained at room temperature (air conditioned 21° C.). Also one can conveniently measure capillary diameter in the tail fin of adult *Triturus,* especially the males, while this is not possible in the heavily pigmented *Pleurodeles* adult. Capillary visibility is good in the larvae of both species.

The animals were maintained either singly in small finger bowls or in groups of 5 to 6 in large finger bowls, and fed with beef liver or live *Tubifex.* The cold-adapted *Triturus* had been kept for several months in stainless steel trays in the refrigerator and fed weekly on live *Tubifex* while at room temperature for a few hours.

Experimental procedures were essentially identical for all animals as follows: Blood was obtained by removing about 1 mm. of the tail tip with a pair of scissors and permitting the tail to bleed directly into a drop of buffered saline on a glass slide. The slide was examined immediately without coverslip and the outlines of about 35 cells traced by means of the *camera lucida.* Placing a coverslip on the preparation resulted in a certain amount of deformation so the practice was abandoned in favor of working quickly before any appreciable drying could take place. Certain precautions that were taken should be mentioned. The slide should be very clean to prevent deformation due to adhesion between the red cell and the glass surface. All margins of the cell must come into focus at the same focal setting, indicating that the cell is resting on one elliptical surface and not oriented at an angle to the plane of observation. Following the tracing of a known linear dimension from a stage micrometer it was possible to determine both the area and the eccentricity of the red cell (area $= \frac{1}{4}\pi ab$, and eccentricity $= a/b$, with a and b the major and minor axes of the cell, respectively). A phosphate-buffered saline (pH 7.4) was found to be a suitable medium for the cells, 0.7% NaCl being approximately isotonic for *Pleurodeles* and adult *Triturus* while 0.6% NaCl was more nearly isotonic for larval *Triturus.*

Capillary measurements were made by lightly anesthetizing the animal by short term exposure to 0.1% chloretone solution, rinsing in tap water and placing the animal on its side on a 5-inch square glass plate. Measurements of capillary diameter were made on the tail fin margin by means of a calibrated ocular micrometer at about 430 ×. Capillaries were identified as the smallest blood vessels constituting a uniform size class when a given portion of the circulation was traced from the arterial to the venous end, and through which the red cells pass in single file. It is important that anesthesia be light as considerable capillary collapse can occur in animals with partially arrested circulation. Measurements were restricted to vessels through which blood was flowing in normal fashion. The measurements on adult *Triturus* were carried out largely on males, not because of any sex differences but because of better visibility in the broader tail fin of the male. The animals recovered from anesthesia in about one hour.

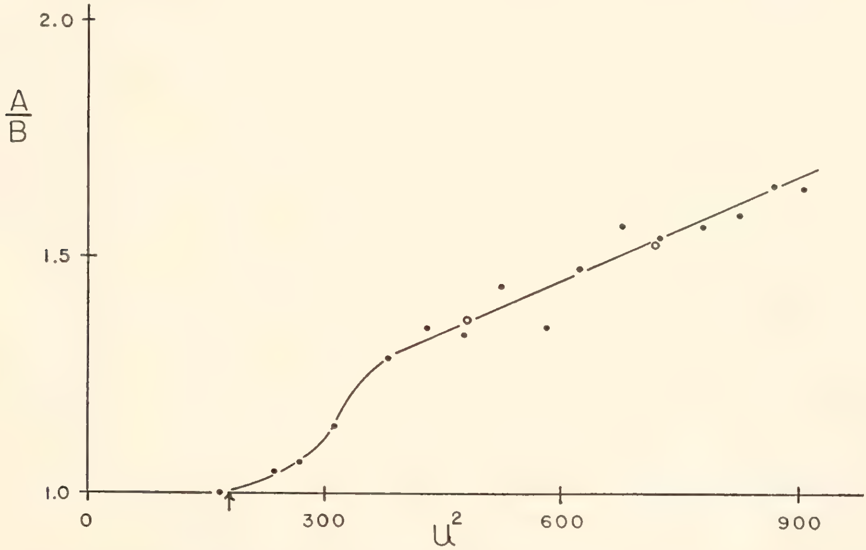

FIGURE 1. The relationship between cell area (μ^2) and cell eccentricity (a/b) in diploid and triploid *Pleurodeles*. The open circles represent the mean values for diploid and triploid blood cells. Other points were obtained by breaking the total sample into classes of 50 μ^2 and plotting the mean values for area and eccentricity within each class. The arrow indicates the mean value for the cross-sectional area of the capillary of the tail fin.

The relationship between red cell form and area was determined as follows with mature larvae of *Pleurodeles*. Approximately 150 cells from diploid animals and an equal number of cells from triploid animals were traced and the eccentricity and area determined for each cell. The mean values for 2n and 3n blood cells were determined from these samples. An additional 30 selected small cells and 30 selected large cells were measured in order to extend the analysis over the widest possible range. The total sample was then arranged in order of increasing cell area, and broken into size classes of 50 μ^2. Within each size class the mean cell area and eccentricity were calculated. Eccentricity (a/b) was then plotted versus area for each size class together with the mean values for 2n and 3n blood (Fig. 1). No significant differences were found between 2n and 3n capillary diameter and the average capillary cross-sectional area for about 40 determinations is indicated by the arrow in Figure 1.

Triturus came from two sources. The males used for the temperature studies and the larvae represent stock originally from Farmville, Virginia. A small group of female animals of uncertain origin were found to have less eccentric blood cells than those of Farmville animals maintained at the same temperature. These females were unusually large and probably represent a genetically distinct population. In that regard it is interesting to note that the values reported for eccentricity in Missouri animals are different from any of the findings in the present study (Davison, 1957). Analyses were made of cell form, cell area, and capillary cross-sectional area for each of the following groups of animals: Farmville males at 21° C., Farmville larvae at 21° C., females of uncertain origin at 21° C., Farmville males adapted to 8.5° C., and the same males during the adaptation period following transfer to 21° C. From 4 to 10 animals were measured from each group. Blood cell findings represent the means of from 90 to 200 measurements and mean capillary size was calculated from 40 to 100 measurements. The same animals were used for capillary and blood cell analyses. The time course experiment following transfer from 8.5 to 21° C. was carried out on a group of 6 *Triturus* on which daily measurements were made for a period of 6 days. The pertinent tabular data derived from these studies appear in Table I.

TABLE I*

Source	Temp. ° C.	Cell area μ^2	Capillary area μ^2	a/b	K in the expression $(a/b)-1=K(A_{\text{cell}}/A_{\text{cap.}})$
Triturus					
Farmville males	21	583	149	1.69	0.177
Farmville males	8.5	576	113	1.99	0.195
Farmville males	21 (6 days)	590	170	1.64	0.184
Farmville larvae	21	523	211	1.44	0.178
? females	21	579	242	1.39	0.163
Pleurodeles					
Mature larvae (2n)	21	482	184	1.36	0.138
Mature larvae (3n)	21	720	184	1.52	0.134

* Standard deviations for cell area were uniformly about 20% of the mean and about 10% of the mean for capillary diameter and a/b.

RESULTS

Cell eccentricity as a function of cell area is plotted in Figure 1. For values of a/b greater than about 1.3, a/b is essentially linear with respect to area and would pass through the origin if extrapolated. For values of a/b less than 1.3, eccentricity rapidly approaches 1 (circular form) as the area of the cell approaches the mean cross-sectional area of the capillary (indicated by the arrow in Figure 1). It is reasonable that if the red cell is no larger than the capillary it can pass through without deformation, accounting for the circular form of the smallest blood cells. Somewhat larger cells may be deformed as they pass through but not actually applied to the wall of the capillary, an interpretation which may account for the curvilinear portion of Figure 1. Still larger cells slide through the capillary with one elliptical surface applied to the capillary wall, with their form determined in accordance with the linear portion of Figure 1. The graphic information in Figure 1 may be given a somewhat more intuitive presentation as a series of forms in Figure 2. The central circle represents the capillary area (184 μ^2) while the surrounding blood cell forms were constructed from the data of Figure 1 at areas of 300, 500, 700 and 900 μ^2. In this and all other reconstructed cell forms the blood cells were assumed to be perfect ellipses in plane view and were first constructed on coordinate paper employing the basic property of ellipses that $x^2/a^2 + y^2/b^2 = 1$ (a and b are the major and minor semi-axes of the ellipse).

Figure 3 illustrates the reconstructed form of larval and adult Farmville *Triturus* cells and capillaries. The larval blood cell is slightly smaller and less eccentric while the larval capillary is larger.

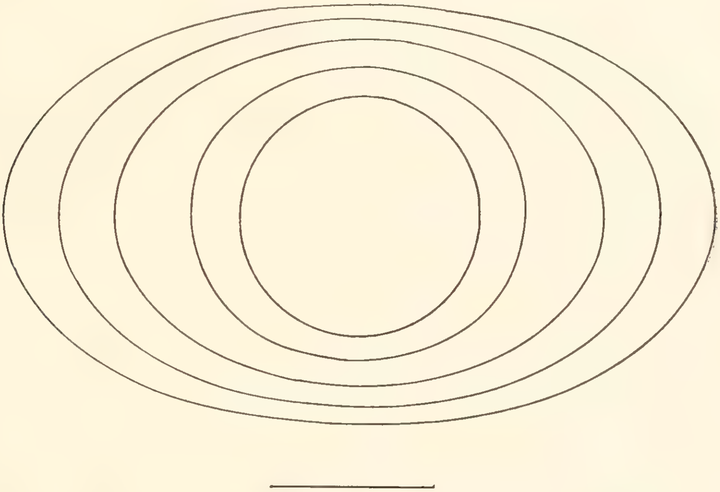

FIGURE 2. The information in Figure 1 was used to reconstruct the form of the blood cells employing the property that $x^2/a^2 + y^2/b^2 = 1$. The central circle is the capillary (184 μ^2). The other figures represent cell forms at 300, 500, 700, and 900 μ^2. The lower line represents 10 μ.

Figure 4 indicates the form differences observed in *Triturus* males maintained at 8.5° C. (left) and 21° C. (center). The right hand figure illustrates red cell form and capillary size in *Triturus* females of uncertain origin (21° C.). It is clear from both Figures 3 and 4 that the larger the capillary the less eccentric the blood cell, a result previously suggested from considerations of the model system. The product of $(a/b) - 1$ and capillary cross-sectional area ($A_{\text{cap.}}$) approximates constancy for adult *Triturus* in which cell area (A_{cell}) is essentially constant. That is:

$$(a/b) - 1 = k/A_{\text{cap.}}$$

The study of eccentricity versus cell area in *Pleurodeles* indicates for the linear portion of the curve (a/b greater than 1.3) that:

$$(a/b) - 1 = k'A_{\text{cell}}.$$

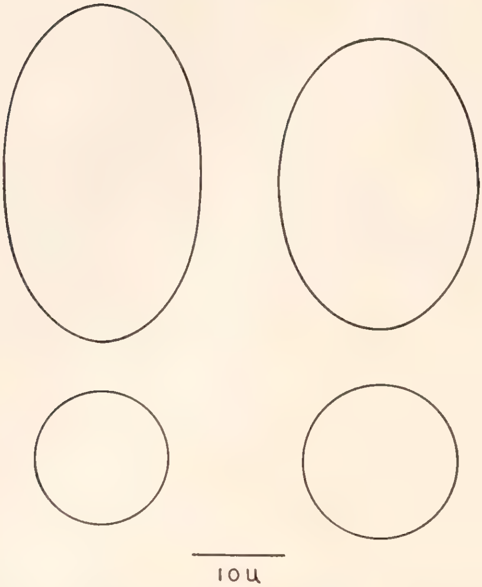

FIGURE 3. The form of the blood cells and capillaries of adult (left) and larval (right) Farmville *Triturus* reconstructed from the tabular data in Table I. The larval blood cell is slightly smaller.

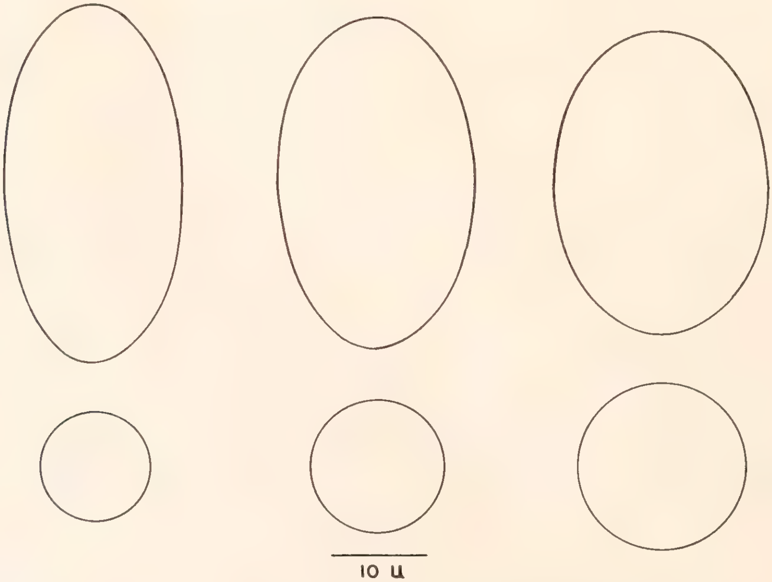

FIGURE 4. The mean forms of blood cells and capillaries in *Triturus* adults constructed from the data in Table I. Farmville males at 8.5° C. (left), Farmville males at 21° C. (center), and females of unknown origin at 21° C. (right). All cells have approximately the same area.

These two expressions may be combined to give an equation relating all three variables: a/b, A_{cell}, and $A_{\text{cap.}}$:

$$(a/b) - 1 = K(A_{\text{cell}}/A_{\text{cap.}}).$$

The extent to which this equation adequately describes the relationship between these variables is clear from the uniformity of the constant K calculated from the data in Table I. *Triturus* values vary from 0.163 to 0.195, while the *Pleurodeles* values are somewhat smaller being 0.138 and 0.134 for diploids and triploids, respectively.

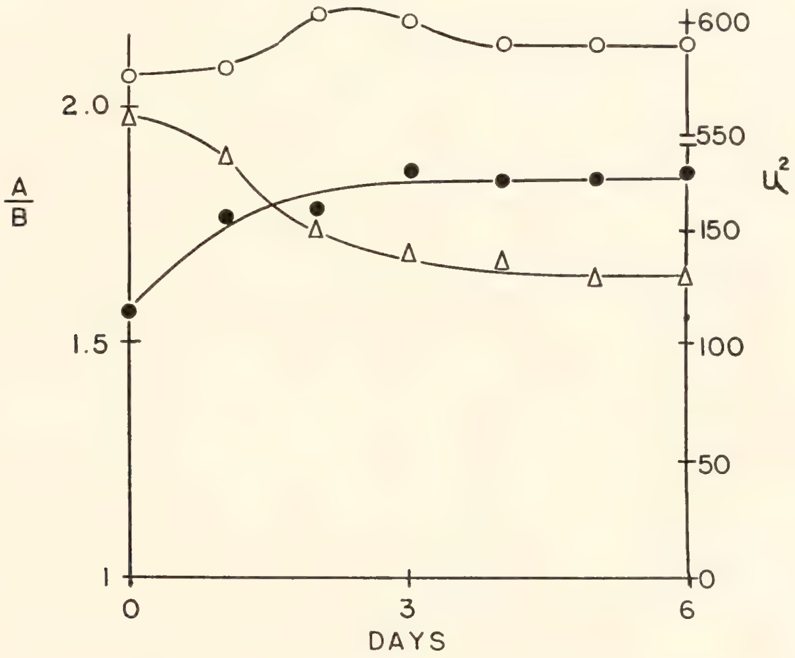

FIGURE 5. The time course of the adaptation from 8.5° C. to 21° C. in Farmville male *Triturus*. Closed circles are capillary cross-sectional area measurements and open circles red cell area measurements (right ordinate). Triangles are eccentricities of the red cells (a/b) (left ordinate). Notice the break in the right ordinate (μ^2).

A point of considerable interest is the time at which red cell form is determined. Two possibilities might be considered. The form of the cell might be determined at the time it first enters the circulatory system with subsequent changes in the capillary environment having no further effect on cell form. If this were true, average cell form should change slowly following a change in capillary diameter, with the total time period for the change equal to the life span of the erythrocyte. On the other hand, if red cell form is plastic, one might expect a more rapid response in red cell form following a change in capillary diameter, a result which would support the concept that red cell form is constantly subject to the forces acting on the cell during its passage through the capillaries. The latter view is clearly favored by the time course data represented in Figure 5. Following transfer of the animals from 8.5 to 21° C., the increase in capillary diameter is complete within 3 days, while the change in red cell form is largely complete within the same period of time. There is, however, a clear lag in the cell form response and a somewhat greater time for the complete form transformation. It is interesting to note that there is a small transient increase in cell area corresponding to the time period when cell form is most rapidly changing. This increase may represent a temporary change in the osmotic properties of the cell dependent on alterations of the cell surface.

Discussion

The quantitative and temporal relationship between red cell form and capillary size clearly supports the concept that the form of the cell is determined, at least in part, by the size of the capillary through which the cell is passing. Alterations in capillary diameter lead to changes in cell form with kinetics supporting a view that the red cell form is plastic and not fixed. Since the reasoning leading to these analyses was influenced by considerations of the model system, a comparison between the model and the biological systems may be useful. I would like to point out first, however, that model systems serve only to guide rationale, and certainly should not be taken as literal representations of biological reality.

(1) There are gross differences between the relative sizes of the "capillary" and the "cell." In the model the cylinder is many times larger than the mercury drop. In the living system the cell is elliptical only if its area exceeds that of the capillary cross-section, while in the model eccentricity steadily increases with drop area since the drop is always in contact with the cylindrical surface.

(2) There is abundant evidence that the interior of the amphibian cell is fluid like the mercury drop. Norris studied the manner in which displaced nuclei returned to the center of the cell and concluded that except for the nucleus the cell interior is liquid with the shape of the cell conferred by an outer envelope (Norris, 1939). Dawson presumed a liquid interior based on the observation of Brownian movement in the cell interior (Dawson, 1928). Based on microsurgical findings, Seifriz described the cell interior as essentially liquid with a plastic and elastic cell envelope approximately 0.8 μ in thickness (Seifriz, 1926). The envelope thickness agrees well with more recent estimates based on polarized light analyses of human red cells (Mitchison, 1953).

(3) The model system is static while the living system is of course dynamic since the cells are constantly moving through the circulatory system. It is of interest to note that although the blood cells are ellipses with symmetrical ends as observed at rest, they clearly do not have this form while passing through the capillaries. The advancing end of the cell is more rounded than the trailing end so that if the cell could be removed and flattened it would approximate a pear shape in plane view. This configuration may also be imitated with the model by tipping the cylinder and permitting the mercury drop to slowly flow along the surface of the cylinder. The dynamic form of the blood cell may be interpreted as further evidence for a fluid red cell interior.

In summary, then, it appears that red cell form is a consequence of physical forces operating between the cell and the capillary wall. In answer to the classical dilemma of whether form determines function or function determines form one may arrive at the inadequacy of either of these alternatives and conclude on the basis of these studies: *form determines form.*

SUMMARY

1. A mercury drop in contact with a cylindrical surface takes the form of a flat elliptical disc. Increasing the volume of the drop causes an increase in the area and eccentricity of the drop but causes no appreciable increase in thickness. With constant drop volume, the larger the cylinder the less eccentric the fluid drop.

2. Analyses of blood cell form and capillary diameter in *Triturus* and *Pleurodeles* disclosed the following relationships. The red cell is circular if its area does not exceed that of the capillary. Eccentricity increases first in a curvilinear and then in a linear fashion as the red cell increases beyond the cross-sectional area of the capillary. Under conditions of essentially constant red cell area, eccentricity is inversely related to the cross-sectional area of the capillary.

3. Based on the experimental findings the following equation may be derived relating red cell area, capillary cross-sectional areas and eccentricity (a/b):

$$(a/b) - 1 = K(A_{\text{cell}}/A_{\text{cap.}}).$$

4. Evidence for the physical nature of the red cell was discussed in relation to the model system.

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THE CONTROL OF REPRODUCTION IN DIPLOPTERA PUNCTATA (BLATTARIA)[1]

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In cockroaches, as in many insect species studied, a hormone released by the corpora allata is necessary for the maturation of eggs (Scharrer, 1946; Engelmann, 1957). A variety of intrinsic and extrinsic factors, such as pregnancy, egg resorption, or involution of the corpus luteum (Lüscher and Engelmann, 1955; Engelmann, 1957), food supply (Scharrer, 1946; Johansson, 1955, 1958; von Harnack, 1958), and probably humidity and temperature are involved in the control of the function of the corpora allata. Furthermore, in some cockroach species, mating accelerates the growth rate of the eggs and, therefore, increases the total number of eggs produced during the lifetime of the animal (Griffiths and Tauber, 1942; Roth and Willis, 1956; Wharton and Wharton, 1957).

In *Diploptera punctata,* a viviparous cockroach, the influence of mating, an extrinsic factor, on egg maturation plays a more dominant role than in related species. In virgins, the maturation of the first batch of eggs takes several weeks or even months (Roth, personal communication), whereas after mating only 10 days are required for egg maturation (Roth and Willis, 1955). Since a variety of factors known to condition the activity of insect ovaries reach the gonads via brain and corpora allata (Scharrer, 1958) it seems probable that the stimuli exerted by the act of mating influence the ovary by the same route.

The present paper deals with the elucidation of the control mechanism governing female reproduction in *Diploptera.* Particular emphasis was given to the study of the pathways by which the mating stimulus reaches the ovary.

MATERIAL AND METHODS

All experimental animals were taken from a stock colony and maintained in finger bowls at room temperature of 22–26° C. They were fed dog chow and water. Daily records were kept of the reproductive activity of the females. The presence of a spermatophore in the bursa copulatrix of the female indicated mating, that of an egg case in the brood sac ovulation.

Experiments such as castration, allatectomy, excision of the gonapophyses, and severance of nerves were performed under CO_2 anesthesia. The nervi corporis cardiaci or the nervi corporis allati were severed either by frontal or occipital approach.

For the histological investigation of the corpora allata and for the determination of the completeness of the severance of nerves an organ complex consisting of brain, corpora cardiaca, and corpora allata was fixed either in Bouin-Duboscq's or in Zenker's fluid. The sections were stained in Gomori's chrome hematoxylin phloxin

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or in aldehyde fuchsin as modified by Halmi. The volume of corpus allatum tissue per one million nuclei was determined by a formula given in an earlier paper (Engelmann, 1957). The data obtained, expressed as mean values for both corpora allata of a given specimen, indicate the degree of activity of the corpora allata; these values were correlated with the observed length of the oocytes.

RESULTS

1. *The cyclic activity of ovary and corpus allatum*

In ovoviviparous or viviparous cockroaches there is no space for additional eggs within the brood sac of the female during the development of the embryos. Egg maturation in the ovaries must, therefore, be temporarily suppressed. Since in other species of cockroaches egg maturation depends on the activity of the corpora allata, we first looked for a possible relationship between the structure of the corpora allata and ovarian activity in *Diploptera* during an interval encompassing the first pre-oviposition, first pregnancy, and second pre-oviposition periods. The results which are reported in the following paragraphs provided a basis for experimental work.

In *Diploptera* mating takes place a few minutes after emergence (Roth and Willis, 1955). Within 9.43 ± 0.22 days thereafter (53 animals observed in our laboratory) the largest oocyte of each ovariole grows from about 0.55 mm. to a size of approximately 1.5 mm. and then ovulates. Large amounts of yolk are deposited in the oocytes during their period of growth.

No eggs ripen in the ovaries during pregnancy, which follows ovulation and which lasts for 75.49 ± 1.47 days (45 animals; see also Willis *et al.*, 1958). There is merely a slight growth of the oocytes, but no yolk whatsoever is deposited until the second or third day before parturition. Then a more rapid growth of the oocytes accompanied by yolk deposition begins, so that on the day of parturition the largest eggs in the ovarioles measure about 0.87 mm. (Fig. 1). Within the next three or four days the eggs grow to full size; 5.98 ± 0.17 days after parturition these mature eggs ovulate (45 animals).

The activity of the corpora allata, as judged by the amount of cytoplasm, correlates with the activity of the ovaries. The relative and absolute amount of cytoplasm in these glands gradually increases during the first pre-oviposition period until a maximum of activity is reached at the fourth day after mating (Fig. 1). Values of less than 0.95 $mm.^3$ of total tissue per one million nuclei indicate that this gland is inactive; more than 1.45 $mm.^3$ tissue per one million nuclei represents a highly active gland. Between these figures there are varying degrees of activity. The cells of the corpora allata maintain a high activity for about four days, during which time most of the yolk is deposited in the oocytes. Shortly before ovulation, a decrease in the activity of the corpora allata is observed, so that on the day of ovulation the corpora allata contain only a small amount of cytoplasm.

During pregnancy, when the ovaries are quiescent, the corpora allata are inactive, as shown by histological criteria (Fig. 1; see also Table II, a); the distribution of the nuclei in the corpora allata is similar to that on the day of emergence, *i.e.*, 0.84 $mm.^3$ gland tissue per one million nuclei. Shortly before parturition an activation of the corpora allata can be observed which gradually increases until the second or third day after parturition (Fig. 1). After reaching a peak of activity, the cytoplasmic content of the glands decreases. The corpora allata are nearly inactive again at the time of the second ovulation.

The second pre-oviposition period is three days shorter than the first. The growth rate of the oocytes, however, is about the same in both periods (Fig. 1). The shortening of the second period is merely due to the fact that yolk deposition in the second batch of oocytes begins already during the late days of pregnancy. The average peak of corpus allatum activity in the second reproductive period is below that of the first, but this difference is statistically insignificant. The reason for the variability of the data is, at least in part, that the onset of the second period of activity is not as clearly defined as that of the first period. Therefore, the corpora allata of some females may have surpassed their highest activity when those of other females, fixed after the same interval, are still maximally active.

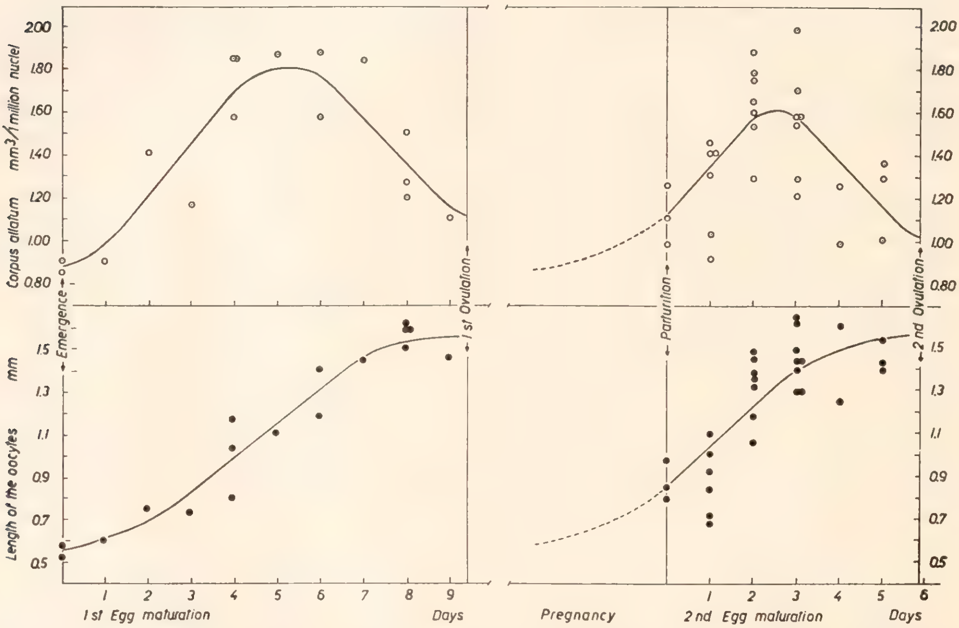

FIGURE 1. Diagram indicating the morphological changes signalling cyclic activity in the corpora allata of *Diploptera* (upper curves), which are correlated with the growth of the oocytes (lower curves) during the first and second pre-oviposition periods.

2. The function of the corpora allata in the adult female

The role of the corpora allata in the ovarian control of *Diploptera* was further elucidated by the removal of these glands. Allatectomy was performed two to four days after mating. In all nine animals thus operated the deposition of yolk in the oocytes beyond the stage characteristic of the day of operation was prevented. Twenty days after mating degeneration of most of the oocytes of the allatectomized animals had begun. It appears that by the fourth day after mating not enough corpus allatum hormone has been released into the circulation to permit the completion of egg maturation. Thus in *Diploptera,* in the same way as in other species of cockroaches, egg maturation depends on the activity of the corpora allata not only during the initial phase, but also during the time when most of the yolk is being deposited in the oocytes.

The activity of the accessory glands of the female genital apparatus also depends on the function of the corpora allata, since no secretory material is detectable in the lumen of these glands in animals allatectomized shortly after mating. There is yet another type of secretory cell that seems to be under the control of the corpora allata as the following observation indicates. Around the sixth day after mating the spermatophore is extruded from the bursa copulatrix (53 animals). At the same time an aqueous fluid appears in the genital apparatus, which probably facilitates the extrusion of the spermatophore. In allatectomized females the spermatophore stays within the bursa, and the genital apparatus remains dry. In four normally mated females, in which for undetermined reasons the corpora allata did not become active, as shown by the failure of eggs to mature, the spermatophores remained in the bursa copulatrix for 18 days at which time the females were fixed for further investigation.

Thus it appears that the hormone released by the corpora allata serves at least three different functions in the adult female of *Diploptera.* It causes the deposition of yolk in the oocytes, it activates the accessory glands of the female genital apparatus, and it stimulates an as yet unknown type of cell presumably present in the bursa copulatrix, whose secretion facilitates the extrusion of the spermatophore. The function of the accessory glands and of the secretory cells in the bursa seems to be regulated directly by the corpora allata, since this mechanism is not disturbed by ovariectomy.

3. The control of the activity of the corpora allata by the brain

In *Diploptera,* as in many other insect species studied, the brain innervates the corpora allata via the nervi corporis allati, which are a component of the nervi corporis cardiaci. Severance of these nerves was attempted in 44 virgins of *Diploptera,* one to 10 days after emergence. Activation of the ovaries, *i.e.,* deposition of yolk in the oocytes, resulted in 39 of these animals, whereas in the five remaining roaches no ovarian activity was found. A histological study of the brain-corpora cardiaca-corpora allata complex of all 44 experimental animals was undertaken to check for the completeness of the severance of the nerves. In 16 of these animals, fixed nine to 33 days after operation, no severance or other disturbance of the innervation of the corpora allata could be detected histologically. This group of 16 virgins includes the five cases mentioned above that matured no eggs; the remaining 11 had matured their eggs. In all of these 16 virgins the corpora allata were inactive at the day of fixation. Obviously, the nerve severance had failed in this group. But how can egg maturation in 11 of these virgins be accounted for? Perhaps in these cases the nerves were squeezed or stretched during the operation, a situation which interfered with nerve conduction and thus led to temporary activation of the corpora allata. Apparently the brain must have resumed the inhibition of the corpora allata a few days later.

In 21 animals of the operated group an interruption of the nerves which innervate the corpora allata could be detected histologically; all of these animals had active corpora allata, as determined by histological criteria. In all, egg maturation took place as a result of the activation of the corpora allata within a period similar to that normally observed after mating. From this it seems that mating does not cause a higher activity of the corpora allata than that which results from severance of the nerves that transmit inhibitory impulses to the corpora allata. One might conclude, therefore, that mating prevents the flow of inhibitory nerve impulses. In the remaining seven animals, which also matured their eggs, the success of the operation could not be determined, because parts of the tissue were lost during the histological procedure. It appears that severance of the nervi corporis allati in virgins results in activation of the corpora allata, which indicates that in unmated young females the brain restrains the function of the corpora allata by way of the nervi corporis allati.

It was of further interest to determine whether the corpora allata severed from the restraining center in the brain remain active permanently or only for a period sufficient to induce the maturation of one batch of eggs. In order to test this point, 15 virgins with severed nervi corporis allati were fixed 14 to 31 days after their first ovulation had taken place. In eight of these animals only one egg maturation had occurred; the size of the next oocytes in line in the ovarioles was about 0.40 mm. at the time of fixation. This size is comparable to the measurements in pregnant females at a similar interval after ovulation. The corpora allata of these eight animals were inactive as determined by histological criteria. The innervation of the corpora allata in these animals seems to have been incomplete. A few intact nerve fibers were observed in the sections. It may be that these had been squeezed and thus prevented from inhibiting the corpora allata for a short time, or these nerve fibers may have regenerated and thus may have resumed inhibition of the corpora allata. This might have been the case also in four additional virgins in which egg maturation had begun a second time, but had not progressed beyond an initial phase; here the largest oocytes measured on the average 0.62 mm. 20 to 30 days after the preceding ovulation, whereas in normal pregnant females, after a similar interval, the oocytes measured only about 0.40 mm. In these operated virgins the resumption of the inhibition of the corpora allata may have taken more time and, therefore, a second growth period of the oocytes had been initiated. Only in three specimens, in which the separation of the corpora allata from the brain was complete at the time of fixation (interval over 40 days), the glands were active (average of 1.32 mm.^{3} gland tissue per million nuclei). In these three animals, the corpora allata had induced the growth of a second batch of eggs to full size (1.50 mm.) within 24 to 30 days after the first ovulation.

Thus, severance of the corpora allata from the restraining center in the brain results in a sustained activity of the glands. This activity persists at least long enough for two successive batches of eggs to mature. In the three animals with completely severed corpora allata the maturation of the second batch of eggs took considerably longer than that of the first. This may have two reasons. In the first place yolk deposition begins when the oocytes measure about 0.55 mm. which means that the oocytes next in line after ovulation have first to grow from about 0.35 mm. to 0.55 mm. This initial growth of the oocytes results in a prolongation of the pre-oviposition period which immediately follows ovulation. A second factor may be a restraining influence coming from the corpus luteum which, during its involution, seems to inhibit the growth of the next oocyte in line, as was reported in *Leucophaea* (Engelmann, 1957).

4. *The activation of the corpora allata by afferent stimuli*

In mated females of *Diploptera* the growth of the oocytes accompanied by the deposition of yolk began about one day after emergence, and was completed within 10 days thereafter (53 animals; see also Table I, a). Among 45 virgins, on the other hand, the shortest interval in which egg maturation occurred was 37 days (one animal); at 60 days after emergence eight additional virgins had ovulated.

TABLE I

Experiments during first pre-oviposition period

	Operation (days after emergence)	Fixation		Number of animals	Corpus allatum (mm.^{3}/1 million nuclei)	Ovary (length of the largest oocytes in mm.)
		(Days after operation)	(Days after emergence)			
a. Control: mated females	—	—	4–9	12	1.49±0.08	1.32±0.08
b. Severance of ventral nerve cord. No mating	1–3	3–23	4–24	8	0.95±0.03	0.60±0.02
c. Severance of ventral nerve cord followed by mating	1	6–20	7–21	8	0.85±0.02	0.61±0.02
d. Artificial mating stimuli	5–19	3–14	17–26	8	1.03±0.08	0.72±0.04
e. Excision of gonapophyses	1–5	8–11	9–14	12	1.33±0.14	1.11±0.10
f. Control: virgin females	—	—	5–31	8	0.84±0.02	0.59±0.01

Numbers following ± are standard errors.

Of the remaining 36 animals 13 virgins ovulated within a period of two to five months, whereas 14 animals had not ovulated when they either died or were discontinued for other reasons. Nine virgins had not ovulated even after five months. It is not definitely known whether or not all virgins eventually produce mature eggs without mating. It is obvious, however, that mating is essential for a normal growth rate of the oocytes maturing after emergence.

One may now ask, whether mating is equally essential to induce the normal growth rate of eggs maturing after the first parturition. Therefore, 24 females were isolated at parturition. The next ovulation occurred 5.83 ± 0.28 days thereafter. In eight additional females, in which mating was allowed to take place on the first day after parturition, the pre-oviposition period took 5.75 ± 0.37 days. Thus, mating does not accelerate the growth of the eggs maturing after parturition. From this it appears that giving birth may serve as a stimulus replacing that of mating from the induction of egg maturation at a normal rate. Furthermore, there is sufficient viable sperm stored in the spermathecae from the mating before the first pregnancy, so that additional batches of eggs can be fertilized.

If parturition activates the corpora allata and consequently the ovaries, how is it possible that yolk deposition in the second batch of oocytes begins a few days before parturition (Fig. 1)? The following observation may give an answer to this question. During the late days of pregnancy, when the abdomen of the female is distended by the ootheca, the animal rhythmically contracts the abdomen. The resulting movements of the egg case in the genital apparatus presumably initiate the activity of the corpora allata occurring before parturition. The observation that mating, as well as parturition, and presumably movements of the egg case in the genital apparatus, activate the corpora allata suggests that sensory receptors located in the genital apparatus receive those stimuli. It is likely that movements of the young hatching within the vagina (Roth and Willis, 1955) enhance the activity of the corpora allata resulting from the rhythmical contraction of the abdomen prior to parturition. It is not known, however, whether the initial activation of the corpora allata by the movement of the egg case in itself would be sufficient to bring about complete egg maturation. From the facts reported here and in the preceding paragraphs it appears that in *Diploptera* afferent stimuli are solely responsible for the induction of a normal growth rate of the oocytes in the ovaries.

TABLE II

Experiments with pregnant females

	Operation (days after ovulation)	Fixation		Number of animals	Corpus allatum (mm.^{3}/1 million nuclei)	Ovary (length of the largest oocytes in mm.)
		(Days after removal of ootheca or parturition)	(Days after ovulation)			
a. Control: pregnancy	—	—	4–62	11	0.83±0.02	0.40±0.01
b. Removal of ootheca	0–41	3–31	19–58	13	0.95±0.03	0.56±0.06
c. Removal of ootheca followed by mating (within 4 to 6 days)	35–38	8–15	45–50	5	1.35±0.12	1.20±0.19
d. Control: parturition	—	4–5	—	5	1.10±0.08	1.14±0.06

Numbers following ± are standard errors.

This conclusion was further substantiated by an additional experiment. After the removal of the egg cases from five pregnant females maturation of the next batch of eggs took at least 41 days when no mating was permitted. On the other hand, if mating was allowed after egg case removal, the oocytes matured within 9 days after mating (Table II, c). The time required for the maturation of the eggs after removal of the egg case and subsequent mating, and that after mating following emergence are practically the same. Again, an external factor accounts for the induction of egg maturation at a normal rate.

If stimuli, received in the genital apparatus during mating and parturition, activate the corpora allata, a transmission of these stimuli to the brain and corpora allata via the ventral nerve cord has to be postulated. In order to test this point the ventral nerve cord was severed in 16 virgins 1–3 days after emergence. Eight of these animals were allowed to mate afterwards, whereas another eight animals were kept isolated as controls. None of the operated and mated females showed maturation of the eggs. The corpora allata must have remained inactive and, consequently, in these animals yolk deposition was completely prevented (Table I, c). The same was the case in the eight females, which did not mate after severance of the abdominal nerve cord (Table I, b). From these experiments one may conclude that stimuli received in the genital apparatus during the act of mating, and presumably also during parturition, are transmitted via the ventral nerve cord to the brain and corpora allata.

Further experimental proof for the conclusion that stimuli are received in the genital apparatus seemed desirable. Therefore, a small artificial spermatophore, made of glass, was pushed into the bursa copulatrix of virgins (Table I, d). Most of these artificial spermatophores remained in the bursa copulatrix for the duration of the experiment, but in some cases they were extruded and had to be replaced. A few days after successful implantation of the "spermatophores" the animals were sacrificed in order to check the degree of maturation of the oocytes and to determine histologically the state of the corpora allata. Among eight animals five had begun to deposit yolk in the oocytes. This result is not as clear-cut as that after mating (Table I, a), which might be due to the fact that a glass spermatophore is too smooth to afford sufficient stimulation. Furthermore, normal mating may last up to several hours during which time the movements of the male provide a more efficient stimulation of the genital apparatus than that caused by deposition of a smooth artificial glass spermatophore.

Apparently mating alone, without the deposition of a spermatophore, also induces activity of the corpora allata (Roth, personal communication). On the other hand, from the fact that yolk deposition in some of the experimental animals was induced by artificial mating, it appears that mechanical stimulation during the act of mating or during parturition tends to activate the corpora allata and in turn to induce maturation of the oocytes.

As stated above, it was thought that sensory receptors on the gonapophyses of *Diploptera* are involved in the perception of these mechanical stimuli. For this reason in 12 virgins all three pairs of gonapophyses were excised in order to prevent the perception of stimuli, when mating was allowed afterwards. However, none of the virgins thus operated upon accepted a male within the duration of the experiment. Nevertheless of the 12 operated females, 10 were found to have deposited yolk in the oocytes after a few days; in some females the eggs had reached nearly full size (Table I, e). Probably the afferent nerves were stimulated by the excision of the gonapophyses and this resulted in activation of the corpora allata and of the ovaries. The conclusion that sensory receptors on the gonapophyses receive the stimuli during the act of mating or parturition seems, therefore, justified. This experiment does not exclude the possibility that other parts of the genital apparatus supplied with sensory receptors may also be involved in the perception of mating stimuli. As the following experiment shows, this proved actually to be the case. In three virgins with gonapophyses excised shortly after emergence mating eventually took place 21, 28, and 52 days after operation. Nine to ten days later, *i.e.,* after an interval normally required for egg maturation in mated females, ovulation took place. Thus, we conclude that sensory receptors on the gonapophyses and on other parts of the genital apparatus receive the stimuli exerted by mating and parturition.

5. *The effect of mature eggs on the corpora allata*

In other species of roaches mature eggs seem to exert an inhibitory effect on the corpora allata (Engelmann, 1957). The question arises whether in *Diploptera* mature eggs, either before ovulation or during embryonic development in the brood sac, likewise inhibit the corpora allata in their function. For this reason, the egg cases were removed from the brood sac during anesthesia in 13 females of *Diploptera,* at different stages of pregnancy. These animals were kept isolated to prevent mating and were fixed three to 31 days thereafter in order to check the degree of activity of the corpora allata and of the ovaries. None of these 13 females matured their eggs in the ovaries within the tested period (Table II, b). Additional five females, in which the oothecae were removed three to five days after ovulation, were kept until the next ovulation occurred. This took place 42, 51, 54, 57, and 71 days after the removal of the oothecae. Thus, the period required for egg maturation after removal of the egg case is roughly comparable to that observed in virgins. In other words, in this experimental series in which appropriate afferent stimulation was lacking, the corpora allata were retarded in their activity, but not completely suppressed.

By contrast, egg maturation is inhibited throughout the period of pregnancy which in *Diploptera* lasts 75 days. Therefore, the absence of mating or equivalent stimuli alone would not account for this complete state of quiescence in the ovary which in turn signals complete inactivity of the corpora allata. An additional inhibitory action seems to come from the ootheca and, in analogy with the known situation in *Leucophaea,* it may be due to a humoral factor.

As to the mechanisms which, in the absence of appropriate stimuli such as mating, keep the corpora allata of adult females of *Diploptera* partially suppressed, nothing can be added to the already discussed inhibitory nervous link between brain and corpora allata. It may well be that the factor eliciting this inhibition acts independently of the inhibitor released by the egg case.

Further support for the concept that a restraining factor from the eggs is not the sole or even primary afferent link governing the inhibition of the corpora allata of *Diploptera* was obtained by castration experiments Eleven females were ovariectomized two to three days after mating. Yolk had already been deposited in the oocytes, which indicated that the corpora allata had been activated by mating. Twelve to 44 days after this operation the females were fixed in order to check histologically the activity of the corpora allata. The glands were completely inactive as indicated by the calculation of an average of 0.86 mm.^{3} of gland tissue per million of nuclei. From this it again appears that an as yet unknown mechanism residing outside the ovary inhibits the corpora allata. These findings in *Diploptera* are in contrast to observations in several other species of insects including *Leucophaea* (von Harnack and Scharrer, 1956) where ovariectomy results in hyperactivity of the corpora allata.

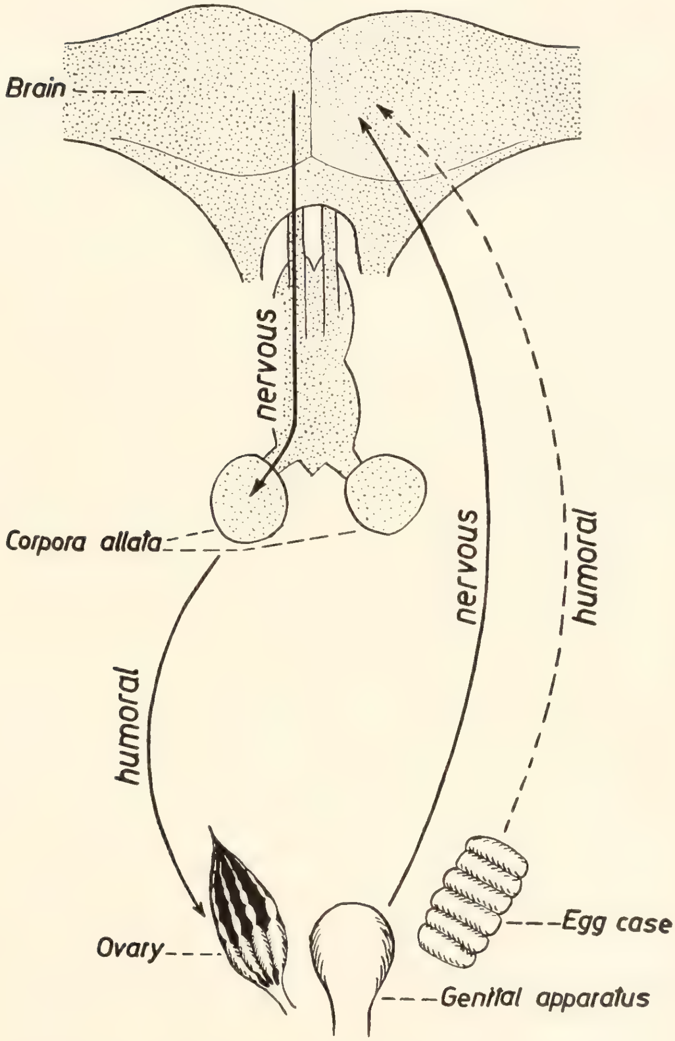

FIGURE 2. Diagram illustrating the control of reproduction in *Diploptera*. Stimuli exerted by mating or parturition, received in the genital apparatus, are transmitted to the brain where they inhibit the normal flow of restraining impulses from the brain to the corpora allata. The corpora allata then release gonadotropic hormone which induces egg maturation. During pregnancy, complete inhibition of the corpora allata is accomplished, presumably by a humoral principle originating in the developing eggs. For further details see text.

DISCUSSION

The control of egg maturation in the viviparous cockroach, *Diploptera,* depends on the following sequence of events (Fig. 2): Stimuli exerted by mating, which are received by sensory receptors on the gonapophyses or other parts of the genital apparatus, ascend via the ventral nerve cord to the brain and act to inhibit the normal flow of restraining impulses from the brain to the corpora allata. The corpora allata then release a hormone that induces the maturation of the eggs. Parturition provides stimuli adequate for eliciting egg maturation in the second or subsequent cycles, and no mating is then required. In virgins, the corpora allata seem to be completely inhibited at first, but become slowly activated after some time and eventually induce egg growth. During pregnancy another controlling factor originating in the ootheca makes the inhibition of the corpora allata complete.

As in *Leucophaea* (Scharrer, 1952; Engelmann and Lüscher, 1956), and in *Oncopeltus* (Johansson, 1958), the brain in *Diploptera* restricts the function of the corpora allata by way of the nervi corporis allati. This can be concluded from the fact that severance of these nerves in the species mentioned results in persistent activity of the glands.

The fact that the brain inhibits the corpora allata in *Leucophaea* and *Diploptera* at certain periods suggests that extrinsic and intrinsic "information" is integrated in the brain and then passed on as messages to the corpora allata. In *Leucophaea* the presence of mature eggs in the brood sac causes the brain to inhibit the corpora allata by way of the nervi corporis allati during pregnancy. No eggs mature in the ovary during this period. On the other hand, in *Diploptera,* the brain converts stimuli received in the genital apparatus with the result that inhibitory impulses to the corpora allata cease. The corpora allata then become active and induce egg maturation. In both cases the brain seems to be the regulating center for the function of the corpora allata, and there is evidence that also in other insect species the brain functions as a controlling center for a variety of different endocrine functions (Wigglesworth, 1934; Scharrer, 1958).

Extrinsic factors such as mating and parturition, are particularly important for the proper control of the reproductive cycles in *Diploptera.* In other species of cockroaches, at least one of these factors, namely mating, is involved in reproduction. In *Periplaneta americana,* for instance, mating accelerates growth of the eggs and consequently more eggs are produced during the animal's lifetime if it is permitted to mate (Griffiths and Tauber, 1942; Roth and Willis, 1956). In this species, however, the influence of mating seems less pronounced than in *Diploptera.* The same is true for *Leucophaea maderae* where mating also enhances the activity of the corpora allata during the first pre-oviposition period. Egg maturation in this species is accelerated by mating only by about one-third of the period required in virgins (Engelmann, unpublished data) in contrast to the observation in *Diploptera* where the acceleration of egg maturation upon mating amounts to at least five times; on the average even more.

In the second and subsequent reproductive cycles of *Diploptera* the stimuli normally occurring in parturition are essential in replacing those of mating. In *Periplaneta* and in *Leucophaea,* on the other hand, no comparable stimulation seems to be required as is shown by the following observations. In females of *Periplaneta,* isolated after their first mating, egg maturation in successive batches took only slightly longer than that in females which were continuously kept with males (Griffiths and Tauber, 1942). In this species parturition does not occur and, therefore, mating would have to replace the effect of giving birth as described in *Diploptera.* In contrast to the situation in *Diploptera,* mating in *Leucophaea* after removal of the egg case did not shorten the time needed for egg maturation (Engelmann, unpublished data). At the present time no explanation can be given for the fact that in *Periplaneta* and in *Leucophaea* mating influences egg maturation only during the first pre-oviposition period and not thereafter. From this it seems that *Diploptera* is unique among cockroaches with respect to the degree to which stimulation of the corpora allata and consequently activation of the ovaries depends on afferent nervous stimuli.

In females of *Diploptera* the normal mating act can be simulated by "artificial mating," a fact which indicates that mechanical stimulation of parts of the genital apparatus is the essential factor involved. It might be worth mentioning in this connection that in some mammals such as the ferret, rabbit, weasel, cat, mating likewise is essential for the completion of egg maturation and ovulation. In the cat, ovulation can be brought about even by artificial stimulation of the cervix uteri by means of a glass rod (Greulich, 1934; Porter *et al.*, 1957). Here, upon mechanical stimulation of an area in the genital apparatus, the brain activates the hypophysis, which in turn induces ovulation by releasing gonadotropic hormones; this is analogous to the situation in *Diploptera*. One should point out that in the cat maturation of the egg nucleus and the rupture of the follicle is induced by mating, whereas in *Diploptera* it results in deposition of yolk in the oocytes. These are two fundamentally different processes related to different reproductive mechanisms, but both are induced by mating and both finally lead to ovulation.

In *Diploptera,* as in *Leucophaea,* we observe alternating activity and inactivity of the corpora allata correlated with periods of egg maturation and of quiescence of the ovaries. In this respect the reproductive patterns of two related species are strikingly similar, but as was shown the regulation of these reproductive cycles is achieved in different ways. In the female of *Diploptera* the corpora allata seem to be maintained at a low level of activity. For every activation of these glands, sufficiently high to induce egg maturation within a normal period of time, a stimulation of the genital apparatus has to take place. On the other hand, in most virgins of *Leucophaea* the corpora allata appear to be only slightly inhibited during the first pre-oviposition period. During pregnancy, in *Diploptera,* a substance furnished by the mature eggs in the brood sac inhibiting the corpora allata seems not to be the primary restraining principle. In pregnant *Leucophaea,* however, such a substance originating in the eggs plays a dominant role in the control of the corpora allata (Engelmann, 1957). Thus, in both related species we find humoral as well as nervous afferent stimuli involved in the regulation of reproduction. They seem to operate independently of each other. They are effective in both species, but in *Diploptera* the nervous factor is the more important, whereas in *Leucophaea* the reverse is the case.

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SUMMARY

1. In the viviparous roach, *Diploptera,* the factors controlling the maturation of the eggs were studied during an interval encompassing the first pre-oviposition, first pregnancy, and second pre-oviposition periods. During egg maturation the corpora allata become active as judged by histological criteria (increase of cyto-

plasmic content); they become and stay inactive during pregnancy. Ovarian inactivity after allatectomy also demonstrates that active corpora allata are responsible for the deposition of yolk in the oocytes. In addition, the corpora allata control the activity of the accessory sex glands, and of an as yet unknown type of cell presumably located in the bursa copulatrix, the secretion of which facilitates the extrusion of the spermatophore.

2. In virgins, the brain inhibits the function of the corpora allata by way of the nervi corporis allati, and thus prevents or delays the maturation of the eggs. Severance of these nerves results in a sustained activity of the corpora allata with the result that several successive batches of eggs mature.

3. In females which have not yet borne young, mating is essential for a normal rate of egg maturation. The effect of mating can be replaced by parturition which provides adequate stimuli to induce egg maturation. Thus, in an animal that has just given birth, mating does not step up the growth rate of the eggs. The stimuli exerted by mating or parturition are transmitted to the corpora allata by way of the ventral nerve cord and the brain.

4. Artificial mating (mechanical stimulation with a small glass spermatophore) also results in activation of the corpora allata and in subsequent maturation of the eggs. Similarly, excision of the gonapophyses induces egg maturation, presumably because the afferent nerves are stimulated by this procedure. It seems likely that sensory receptors on the gonapophyses and possibly on other parts of the genital apparatus receive the stimuli exerted by mating and parturition.

5. The corpora allata of adult females of *Diploptera* are restrained to the extent that egg maturation occurs only after considerable delay unless this inhibition is eliminated by appropriate afferent stimuli. Complete inhibition of the corpora allata, and thus of the ovaries, during pregnancy is achieved by the eggs developing in the brood sac.

6. Ovariectomy does not result in sustained activity of the corpora allata as is the case in some other insect species.

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SOMITE GENESIS IN THE CHICK. II. ANALYSIS OF NUTRIENTS FROM YOLK[1]

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A previous communication has disclosed that there are a number of components in the albumen of the avian egg which collectively are used by the embryo in the development of somites (Fraser, 1957). These components were indicated to be such small molecular forms as glucose, alanine and a heat-labile substance. Moreover, the protein moiety of the albumen may possibly be used to a limited extent as well in this function.

Because the yolk provides the natural environment of the early chick blastoderm, it seemed advisable to assay this portion of the egg for its nutritional value with respect to the formation of somites. Studies by Needham (1931) and Romanoff and Romanoff (1949) have indicated that the yolk of the avian egg is chemically complex, containing such diverse molecular species as proteins, phospholipids, coenzymes, amino acids, etc.

It has become apparent from many studies that the embryo does not use indiscriminately all of the materials made available to it at any one period during development. For example, the transitional nature of energy sources has been pointed out by Needham (1950). Fraser (1956) has shown that the early blastoderm will starve on a fat diet, although there is much of this food substance present in the unincubated egg.

With respect to the utilization of certain nutrients for specific morphogenetic events, there exists but little information. Wilde (1955) has revealed the use of various portions of the phenylalanine molecule by neural crest cells of the amphibian in melanogenesis. Similarly, the development of the heart and of the brain of the chick embryo have been shown to have different sugar requirements (Spratt, 1950). The present paper represents an attempt to assay the yolk of the chicken egg for its nutritional value, specifically in somite genesis. Other papers to follow will cover other facets of this morphogenetic event.

MATERIALS AND METHODS

While a few of the eggs used in the present investigation were from Rhode Island Red chickens, most were from White Leghorn hens. Nutritionally, the two breeds appear equivalent, although the rate of development of the former is perceptibly slower during the earliest phases of embryogenesis. The eggs were stored at 18° C. until incubated, in all instances within a week after they had been layed. Eggs were incubated at 38.0° C., while explants were cultured at 37.8° C.

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General methods for the culture of the early chick blastoderm have been outlined previously (Spratt, 1947; Fraser, 1957). Definitive primitive streak (DPS) blastoderms were removed from the eggs following approximately 21 hours of incubation, and placed on semi-solid media. After camera lucida diagrams had been made of them, the embryos were incubated for a period of 22 hours, after which time diagrams were again made for the purpose of recording the extent of development. The embryos were then fixed on the gel with Gerhard's fixative and prepared for whole mounts. Delafield's hematoxylin was used as a stain. Greater accuracy could be obtained in counting somites in stained preparations. Approximately six hundred explants were cultured in the course of this investigation.

Preparation of Media

The reader is referred to a previous communication (Fraser, 1957) for an outline of the general procedure in preparing the media used in this study.

Yolk dialysate. One hundred and fifty ml. of yolk from unincubated fertile eggs were dialyzed against 75 ml. of chick Ringer's solution in cylinders for two days at 5° C. The dialysate was then collected and stored under refrigeration until used.

Dialyzed yolk. One hundred ml. of whole yolk were dialyzed in the cold for three days against large volumes of the Ringer's solution. Dialysis proceeded in 10-liter flasks, the saline contents of which were changed on three occasions. The contents of the casings, representing the large molecule fraction of whole yolk, were then collected for use.

Boiled dialysate. Fifty ml. of freshly prepared yolk dialysate were gently refluxed for 10 minutes prior to use.

Ether partition of yolk dialysate. Fifty ml. of freshly prepared yolk dialysate were shaken on three occasions with 15 ml. of redistilled ethyl ether in a separatory funnel. The pooled ether phases were condensed at 45° C. under vacuum to near dryness. Five ml. of chick Ringer's were added and the remaining ether was distilled. Ether was removed from the aqueous phase in the same manner.

Acid hydrolysis of dialyzed yolk. The preparation of amino acids by the hydrolysis of dialyzed whole yolk used in the present study has been described by Block *et al.* (1958). Twenty ml. of dialyzed yolk were boiled under reflux with 40 ml. of 8 N sulfuric acid for 20 hours. To this, hot saturated barium hydroxide was added until a pH of 11 had been reached. After distillation *in vacuo* had removed the free ammonia, the excess barium was precipitated by an equivalent amount of 1 N H_2SO_4. The $BaSO_4$ was centrifuged off and the supernatant fluid reduced in volume to dryness. The residue was then taken up in 10 ml. of chick Ringer's solution for use.

Chromatographic Analysis of Yolk Dialysate

Twenty ml. of the yolk dialysate, prepared as indicated above, were reduced in volume to zero under mild heat from an infra-red lamp, and while agitated by a jet of air. The excess salt was removed by solvent extraction as outlined by Harris (1953). Drops of the concentrated aqueous phase, following ether extraction, were applied to Whatman No. 1 paper, measuring 18 by 22 inches. Two-dimensional chromatograms were prepared by descending chromatography with water-saturated phenol as the first solvent and lutidine-collidine-water (3:1:1 by volume) as the second. After drying, the papers were sprayed with a 0.1% ninhydrin solution in 95% ethanol. On drying in an oven, the spots that appeared were compared with those on the amino acid map of Dent (1948). Similar chromatograms were made of the dialysate concentrate following oxidation with hydrogen peroxide, a procedure that is necessary for the identification of cysteine.

Figure 1 illustrates the identification of the free amino acids in yolk. In all there are thirteen such compounds readily distinguishable by this assay method. Spots numbered 1, 2 and 3 appeared only from a preparation following oxidation.

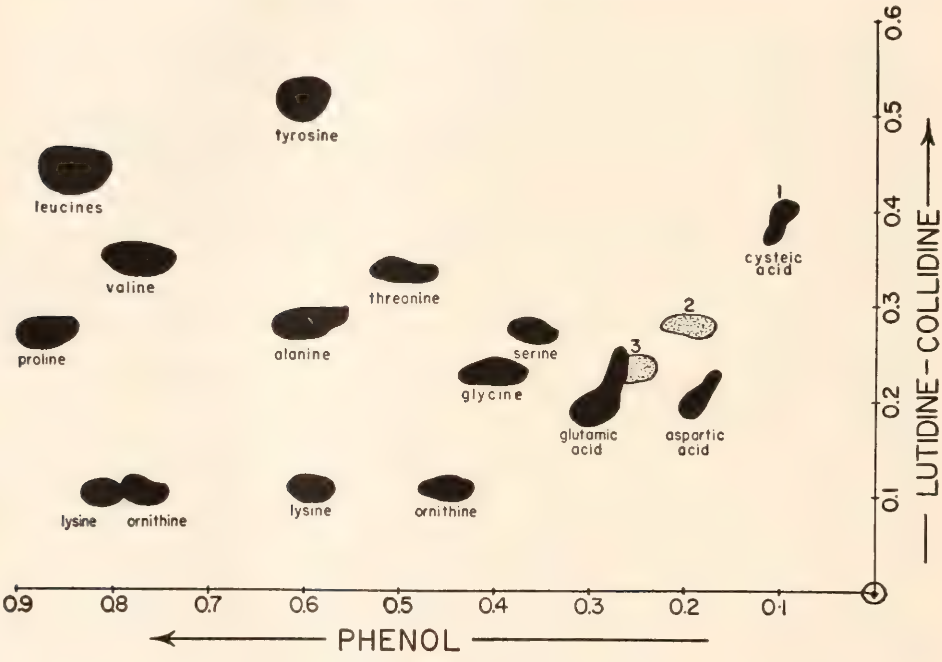


FIGURE 1. Two-dimensional chromatogram showing the amino acids detected free in yolk dialysate.

Grey colored spots indicated by numbers 2 and 3 do not coincide with any shown on Dent's map, and hence are unidentified at this time. The presence of cysteic acid, however, indicates the presence of free cysteine.

BARIUM AND ALCOHOL FRACTIONATION OF YOLK AND ALBUMIN DIALYSATES

To establish more precisely the requirements of the young chick blastoderm from yolk and albumen in the formation of somites, a barium and alcohol fractionation of these food sources was undertaken. The procedure has been indicated in a general way by Harris (1953).

The pH of 15 ml. of yolk dialysate was brought to 8.2 by the careful addition of 0.1 M NaOH. To this were added 2 ml. of 1 M $BaCl_2$, the pH of which had previously been adjusted to 8.2. The resulting mixture was placed in the freezing compartment of a refrigerator for 30 minutes and then centrifuged. The pellet was washed with five drops of $BaCl_2$ (pH 8.2) and then brought into solution with a small amount of doubly distilled water. To this was added a stoichiometric amount of 1 M Na_2SO_4 to precipitate all of the barium. The resulting supernatant was retained as the *"barium insoluble"* fraction.

The supernatant from this procedure was further fractionated by ethanol. The barium-soluble solution obtained was treated with sufficient molar sodium sulfate to precipitate all of the barium. The supernatant was reduced in volume to 10 ml. and chilled. To this were added 40 ml. of chilled absolute ethyl alcohol, thus bringing the concentration of the alcohol to 80 per cent. After further chilling for 30 minutes the preparation was centrifuged, and the pellet (the *"barium-soluble, alcohol-insoluble"* fraction) was washed with 5 drops of cold 80 per cent ethanol. The supernatant from this fractionation is termed the *"barium-soluble, alcohol-soluble"* fraction.

Table I

Assay of barium and alcohol fractions of yolk and albumen for nitrogen and phosphorus

Fraction	Yolk			Albumen		
	P (μg./ml.)	N (μg./ml.)	P/N	P (μg./ml.)	N (μg./ml.)	P/N
Barium-insoluble	104	10	10.40	3.2	8	0.40
Barium-soluble, alcohol-insoluble	10.8	9	1.20	12.5	26	0.48
Barium-soluble, alcohol-soluble	8.8	664	0.01	8.6	98	0.09
Whole dialysate	129	742	0.17	25.7	128	0.20

The three fractions thus obtained were placed individually in Stender dishes and the volume of each reduced to zero with mild heat and air agitation. Excess salt was removed and the volume again reduced to zero. To each were added 4 ml. of chick Ringer's solution and the resulting fractions set aside for incorporation into media.

A similar fractionation was carried out on albumen dialysate.

Assay of Barium and Alcohol Fractions for Phosphorus and Nitrogen Content

Umbreit *et al.* (1949) have listed the phosphorylated intermediates known to be present in the fractions derived from barium and alcohol treatment. In order to determine the relative concentrations of the various esterified compounds in the fractions used in the test media, an analysis was made on the fractions for nitrogen and phosphorus.

The results of such an analysis are shown in Table I. Total phosphorus was determined by the method given by Umbreit *et al.* (1949) after Fiske and Subbarow (1925). The nesslerization method of Koch and McMeekin (1924) for nitrogen was employed. Phosphorus and nitrogen determinations were made, as indicated in the table, on the whole dialysate, the "barium-insoluble," "barium-soluble, alcohol-insoluble," and "barium-soluble, alcohol-soluble" fractions. Preparations from both yolk and egg white were assayed.

CONTENTS OF THE MEDIA

The contents of the media used in the present investigation are given in Table II, along with the amounts of each expressed in milliliters. Phenol red, in concentration of 0.01 per cent, was used as an inside pH indicator. Moreover, this dye also serves by coloring the medium, thus making it easier for the observer to see the explanted embryos. The preparation of the penicillin-streptomycin solution used to prevent bacterial growth, and the phosphate and bicarbonate buffers has been described previously (Fraser, 1956). Throughout the period of culture the pH of the media remained near 7.6. The concentration of the agar used in preparing the semi-solid gels was 250 mg.%. The Ringer's solution was prepared in accordance with the formula given by Spratt (1947), with NaCl content reduced to 123 millimolar (Howard, 1953). In previous communications (Fraser, 1956, 1957) the details of preparing the media have been given. When the volume of the medium was small, 4 or 8 ml., it was poured into depression slides supported on cotton rings within petri dishes. The media of larger volume were placed in watch crystals held in similar fashion. The cotton rings supporting

TABLE II

Components of media used in the experiments and somite development in explanted chick embryos

Test material	Volume of test material	Ringer	Phenol red	Penicillin streptomycin	Phosphate buffer	Bicarbonate buffer	Total volume	No. embryos	Aver. no. pairs somites ± stand. error
None	—	33	2	2	2	1	40	25	0
Whole yolk	20*	23	2	2	2	1	50	28	7.0 ± 0.6
Yolk dialysate	20	13	2	2	2	1	40	56	7.4 ± 0.3
Dialyzed yolk	20*	23	2	2	2	1	50	42	0
Dialyzed yolk + glucose	28**	15	2	2	2	1	50	22	0
Boiled yolk dialysate	20	13	2	2	2	1	40	45	2.0 ± 0.5
Dialyzed yolk hydrolysate	2	1.3	0.2	0.2	0.2	0.1	4	30	0
Dialyzed yolk hydrolysate + glucose	2.8***	0.5	0.2	0.2	0.2	0.1	4	38	3.2 ± 0.5
Ether-soluble fraction	2	1.3	0.2	0.2	0.2	0.1	4	30	0
Saline-soluble fraction	20	13	2	2	2	1	40	32	7.9 ± 0.3
Free amino acids + glucose	17†	16	2	2	2	1	40	41	2.5 ± 0.3
Barium-insoluble fraction of yolk	3.5††	3.1	0.4	0.4	0.4	0.2	8	48	1.5 ± 0.2
Barium-soluble, alcohol-insoluble fraction of yolk	3.5	3.1	0.4	0.4	0.4	0.2	8	52	0.4 ± 0.2
Barium-soluble, alcohol-soluble fraction of yolk	3.5	3.1	0.4	0.4	0.4	0.2	8	40	1.8 ± 0.4
Barium-insoluble fraction of albumen	3.5	3.1	0.4	0.4	0.4	0.2	8	38	0
Barium-soluble, alcohol-insoluble fraction of albumen	3.5	3.1	0.4	0.4	0.4	0.2	8	45	2.2 ± 0.5
Barium-soluble, alcohol-soluble fraction of albumen	3.5	3.1	0.4	0.4	0.4	0.2	8	36	1.0 ± 0.3

* Twenty-five ml. of whole or dialyzed yolk were shaken with 50 ml. of Ringer's. Twenty ml. of the froth-free liquid were decanted for use.

** Eight ml. of 1% glucose plus 20 ml. of dialyzed albumen prepared as above.

*** Four-fifths ml. of 1% glucose plus 2 ml. of dialyzed yolk acid hydrolysate.

† One ml. of a 100 mg. % solution of each of the 13 amino acids found free in yolk dialysate plus 4 ml. of a 2% glucose preparation. Final concentrations: each amino acid, 2.5 mg. %; glucose, 200 mg. %.

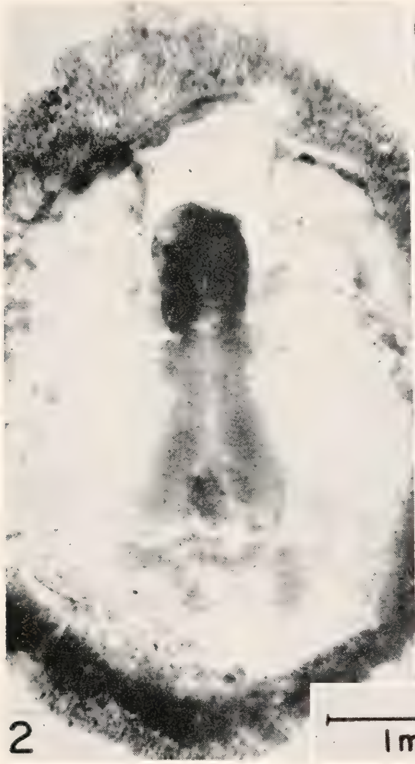
†† The same amount (3.5 ml.) of the barium and alcohol fractions of both yolk and albumen was used.

the containers of media were moistened with sulfadiazine (0.25 per cent) as a further precaution against microbial contamination.

RESULTS AND DISCUSSION

The development of somites in DPS explants cultured on the media outlined above for 22 hours is given in Table II.

One of the most striking observations is that the food materials used in this morphogenetic process are present free in the dialyzable (small molecule) fraction. The same was found to be true when the egg white was assayed (Fraser, 1957). Somites developing in explants on the dialyzable fraction of whole yolk were equal in number to those in explants cultured on the yolk medium itself. On the other hand, no somites formed in blastoderms cultured on the large molecule moiety, even when glucose was added to the medium. This observation would indicate, in contrast to the conclusion of Taylor and Schechtman (1949), that the embryo in culture cannot use native protein alone from either yolk or albumen



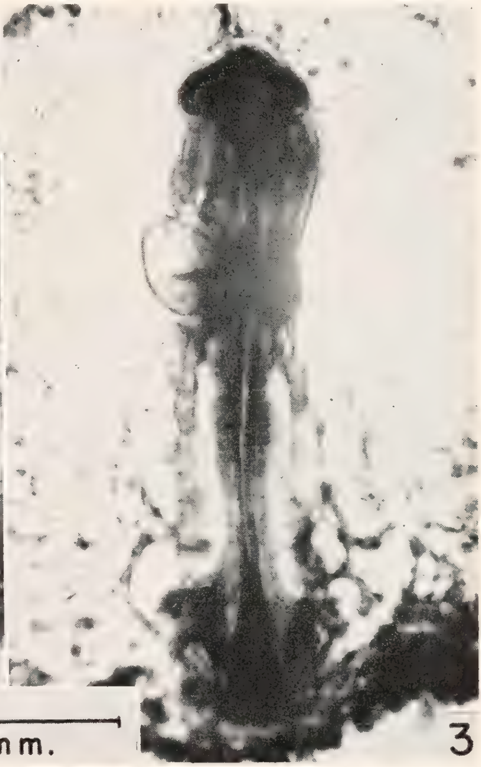


FIGURE 2. Photograph of a DPS blastoderm cultured for 22 hours on dialyzed whole yolk. Note absence of any appreciable development.

FIGURE 3. Photograph of an embryo of similar age cultured for 22 hours on a medium containing yolk dialysate. Note the formation of somites.

for early morphogenesis. Figures 2 and 3 show these results. Blastoderms cultured on dialyzed yolk appear the same as those cultured on dialyzed albumen or on a saline-agar (non-nutrient) medium.

The chick embryo cultured *in vitro* can, however, use the protein moiety of yolk following hydrolysis of the polymers, when supplemented by glucose as an energy source. This observation would suggest that at this stage of development the embryo lacks sufficient proteolytic enzymes for its early morphogenic requirements, and relies almost entirely (if not completely) on molecules of simpler form found free in its environment.

There is, as yet, no complete characterization of the small molecule nutrients used by the chick blastoderm for somite development. Ether fractionation reveals that all materials used are in the aqueous phase. The inability of the very early embryo to use fat as a substrate for development has been noted previously (Fraser, 1956, 1957). It is equally true that some of the somite-forming capacity of yolk dialysate is curtailed by heat. A similar heat-labile fraction has been found in albumen dialysate (Fraser, 1957). There is further support for the presence of a heat-labile component in the observation that somite development is somewhat depressed in explants cultured on a medium containing all of the amino acids found free in yolk.

As one would expect, the bulk of nitrogen, signifying the presence primarily of amino acids, is found in the barium- and alcohol-soluble fraction of both yolk and albumen. In view of the fact that there is some development of somites in explants cultured on the amino acids detected in this portion of the avian egg, it is not surprising that this fraction derived from barium and alcohol precipitation should yield a similar result. This is borne out by observation.

There has been, however, some dispute as to whether the early chick blastoderm can utilize phosphorylated carbohydrate intermediates for its development. Needham and Nowinski (1937) have come to the conclusion that such substances cannot serve as nutrients for the early chick embryo. Novikoff, Potter and Le Page (1948), on the other hand, have demonstrated the presence of phosphorylated intermediates in embryonic chick homogenate. Fraser (1956) has reported the presence of cytochrome oxidase in blastoderms as young as the beginning streak stage. The great importance of glucose in the development of the early chick embryo has been repeatedly demonstrated (Spratt, 1949; Fraser, 1954). Moreover, unpublished work from this laboratory has indicated that only a small portion of carbohydrate metabolism in the young avian embryo is directed through the phosphogluconate shunt. It is evident, therefore, that unless some other, much less likely, mechanism for sugar oxidation exists, carbohydrate must be handled in the conventional phosphorylated manner. If this assumption is true, we might well expect that phosphorylated intermediates, when offered to the embryos, would be metabolized. This contention is borne out by observation that somite development proceeds to a limited degree in explants cultured on media containing these carbohydrate phosphate esters.

Umbreit *et al.* (1949) have listed the various phosphorylated compounds precipitated by barium and alcohol fractionation. According to them, most of these intermediates in carbohydrate metabolism are brought down in the barium-soluble, alcohol-insoluble fraction. Comparison of P/N ratios would indicate that such fractionation precipitates different compounds in albumen than in yolk. The wide discrepancies in phosphate values point to this conclusion. Differences in somite counts in embryos cultured on these fractions add further support. Finally, embryos were cultured on media containing the barium-soluble, alcohol-insoluble fractions of both yolk and albumen dialysates which had been boiled. No somites formed in blastoderms explanted on either of these media. There was no suppressing effect, however, by heat on the barium-insoluble fraction of yolk. We might conclude from this, then, that insofar as the development of somites is concerned, the explanted chick embryo utilizes at least one different component which is present in yolk but not in egg white.

Finally, we should attempt to answer the provocative question why it is that less than the maximal number of somites form in embryos cultured on certain media. Actually the total number of somite pairs found in explants cultured under optimal conditions on any yolk or albumen medium is somewhat less than that seen in embryos grown *in ovo* for a similar period of time. We can therefore conclude that the *in vitro* culture technique falls short in providing the embryo with appropriate environmental conditions other than of a nutritive nature. But insofar as somite development in explanted embryos alone is concerned, such differences must have a nutritional basis, since physical environmental conditions are presumably equivalent.

The experiments in this and the former paper in this series (Fraser, 1957) have shown that the formation of somites in the chick embryo cultured *in vitro* is dependent on a number of chemical constituents in the food supply. We might therefore profitably think that a submaximal number of somites in explants results when one or more of the nutritional requirements for the maximal formation is lacking. This deficiency may then act to limit the number of somites which could develop within a specified period of time. It is only when all of the components needed for this morphogenetic event are present that the maximum number of somites will form.

SUMMARY

1. Definitive primitive streak chick embryos have been cultured *in vitro* on media containing various fractions of whole yolk. Other embryos were grown on agar gels containing fractions derived by barium and alcohol fractionation of yolk and albumen dialysates. Following 22 hours of incubation, the blastoderms were mounted and examined for the development of somites.

2. The nutritional components of whole yolk are all present in the saline-soluble, dialyzable moiety, although the embryo can use the acid hydrolysate of dialyzed yolk, when accompanied by glucose, for this morphogenetic process.

3. The results, derived from an assay of barium and alcohol fractionation of both yolk and egg white, indicate that the early chick embryo can use, to limited extent, certain phosphate esters of carbohydrates in the formation of somites. There appear to be different phosphorylated materials in yolk than in albumen used by the embryo.

4. Insofar as whole yolk utilization is concerned for the formation of somites, there is evidence that the chick embryo grown *in vitro* uses: (1) glucose, (2) the amino acids found free in yolk, (3) a heat-labile, uncharacterized factor, and (4)

certain phosphorylated carbohydrate intermediates. It may also use, but to a very limited extent, the products of proteolysis of yolk proteins.

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PRESENCE OF THE RED EFT WATER-DRIVE FACTOR PROLACTIN IN THE PITUITARIES OF TELEOSTS

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The lactogenic hormone (prolactin), on account of the complexity and diversity of its functions, continues to excite speculation concerning its relation to changing target organs during the course of vertebrate evolution. The role of this hormone in the promotion of lactation in mammals, its effect on the secretory activity of the crop gland in pigeons, and its association with certain aspects of parental care in both mammals and birds are well known. In addition, prolactin is thought to act as a hyperglycemic agent in some higher vertebrates and may be important in regulating various events in the reproductive cycle, such as ovulation and progesterone secretion. Recently, Juhn and Harris (1958) have shown that the lactogenic hormone may participate in the production of new plumage in birds through stimulation of the feather papilla.

The problem of prolactin in the lower vertebrates has been reviewed by Atz (in Pickford and Atz, 1957) with special reference to fishes. There is considerable evidence that this hormone plays a physiological role in the poikilotherms, although direct evidence for its presence is scanty. Blair (1946) found that prolactin (of uncertain purity) stimulated the production of new melanophores in toads, while Pickford and Kosto (1957) have shown that highly purified intermedin-free prolactin promotes melanin synthesis (but not new pigment cell formation) in the partially depigmented melanophores of hypophysectomized killifish, *Fundulus heteroclitus.* The synergic action of prolactin on the melanocyte-stimulating effect of intermedin (new pigment cell formation) was also demonstrated. The acceleration of molting in prolactin-treated newts, *Diemyctylus* (= Triturus) *viridescens,* observed by Chadwick and Jackson (1948), may have resulted from stimulation of the endogenous release of thyrotropin rather than to a specific action of prolactin since the recipients were not hypophysectomized. The lactogenic hormone stimulates the secretion of jelly by the oviducts of anurans (de Allende, 1939; Houssay, 1947 and later papers cited by Pickford and Atz, 1957; de Allende and Orias, 1955). Positive results could be obtained even after castration or hypophysectomy and the gonadotropins were ineffective. The gonadotropic effects of prolactin on the European newts, *Triton cristatus* and *T. alpestris,* and the stimulation of sperm release in male dogfish, *Scyliorhinus caniculus,* reported by Tuchmann-Duplessis (1948, 1949) and Carlisle (1954), respectively, are interesting but require further investigation.

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A most curious and interesting reaction to prolactin was described by Chadwick (1940) in the immature, terrestrial (red eft) stage of *Diemyctylus viridescens.* The red efts migrated to water within a few days after injection of the hormone and developed the olive pigmentation and strongly keeled tail of the aquatic adult. Similar results were reported by Tuchmann-Duplessis (1949) in experiments with immature terrestrial stages of *Triturus alpestris.* In a further study of this problem, Grant and Grant (1956, 1958), using hypophysectomized efts to exclude the reflex release of other pituitary factors, have confirmed that prolactin is directly responsible for initiating the change in habitat preference. Hypophysectomized efts receiving intraperitoneal injections varying from 8 to 0.04 mg. of highly purified prolactin (C. H. Li) migrated to the water compartment of the vivarium but failed to develop the pigment and morphological characteristics of the complete "water-drive syndrome." The total response observed by Chadwick must therefore involve other hormonal factors. In developing a more decisive test for the water hormone, Grant (1958 and unpublished data) has estimated that a minimum dose of 1.4 μg. of prolactin is necessary to elicit the response in 50% of the hypophysectomized efts tested.

Although the indirect evidence cited above is strongly in favor of the hypothesis that prolactin plays a natural role in the physiology of the lower vertebrates, direct evidence for the presence of this hormone in the poikilotherm pituitary is limited and requires confirmation. Leblond and Noble (1937), using the pigeon crop test, obtained weakly positive results of an indecisive nature with pituitary implants of turtle (*Kinosternon odoratum*), frog (*Rana pipiens*) and some species of teleostean fishes (*Ameiurus nebulosus* and *Lepomis gibbosus*) although others were negative. Foglia (1940) found that pituitaries of the toad, *Bufo arenarum,* stimulated the crop gland of the pigeon. Carlisle (in Medawar, 1953) reports that dogfish pituitary contains a factor which promotes lactation in the mammary gland, but the presence of oxytocin may be suspected. More recently Lehrman (in Pickford and Atz, 1957) has demonstrated prolactin-like activity in pollack pituitary brei (*Pollachius virens*) by means of the pigeon crop test. However, negative results were obtained with a lyophilized powder derived from hake, pollack and cod (Wilhelmi, Lot F80x) (Lehrman, personal communication). The data of Fonseca Ribiero and Tabarelli Neto (1943), with alcohol-preserved pituitaries of the teleost *Prochilodus harti,* depend on the validity of the anuran oviduct test. Chadwick (1941), using the red eft test, demonstrated the presence of a "water-drive factor" in the pituitaries of the toad, *Bufo americanus,* the water snake, *Natrix* sp. and the chicken. The pituitaries of two salamanders (*Plethodon metcalfi* and *Desmognathus fuscus*) and of two snakes (*Diadophis* sp. and *Thamnophis* sp.) gave negative results.

The present investigation was undertaken to obtain further information on the distribution of prolactin among the lower vertebrates. Pituitary glands of pollack, *Pollachius virens,* carp, *Cyprinus carpio,* and killifish, *Fundulus heteroclitus,* were tested for the presence of the water-drive factor by means of the red eft test.

MATERIALS AND METHODS

The pituitary material was collected and prepared by one of us (G. E. P.). The wet weight of an average gland varies with the species, the size of the fish, the stage of the reproductive cycle, and possibly also the sex. In any given sample, fishes of both sexes and different size ranges were taken together so that the approximate number of glands injected per eft is less meaningful than the actual weight of the material (Table I). In the case of *Fundulus* it should be noted, however, that unpublished data of B. Kosto have shown that the mean weight of the pituitary expressed in terms of body weight is less in winter (sexual regression) than in spring. The mean index, Pituitary Wt./Fish Wt. in mg. per cent was 0.36 in September and October, 0.51 in April. (Both sexes combined, no significant sex differences were noted.)

Pollack (Lots 1, 2, 3, 4): Glands were collected from fish in the pre-spawning condition at Wilson's Beach, Campobello Island, on June 28, 1954. Fish were brought in in the morning and the glands removed within two hours, wrapped in "Parafilm" in lots of 5 glands each, and frozen immediately. After four years storage in a closed can in the deep freezer, it was found from their appearance that many of the glands were partially desiccated. The average weight was approximately 50% of the expected wet weight (ca. 20 mg.). Therefore, in making up dilutions for comparison with carp and *Fundulus* pituitaries (not semi-desiccated) it was assumed that 100 mg. ═ 200 mg. wet weight. Pollack glands were weighed in lots of ca. 100 mg. and homogenized in 2 ml. of 0.6% NaCl. The brei was kept frozen until used. *Carp* (Lots 5, 6, 7): The glands were taken from fish collected in the Connecticut River on October 22, 1957. Although late in the season, some of the males had flowing sperm and many of the females had well-developed ovaries. After eight months storage in closed vials in the deep freezer the glands were weighed in lots of 200 mg. each (9–12 glands) and homogenized, as in the case of the pollack material, in 2 ml. of 0.6% NaCl.

TABLE I

Doses and calculated numbers of pituitaries involved for each lot of teleost material tested

Lot numbers	Number of test animals each lot	Total dose in mg.	Approx. number of donor pituitaries per dose
Pollack: No. 1	4	10.0	0.8
	2	20.0	1.8
No. 2, 4*	4	10.0	1.1
	2	20.0	2.2
No. 3	4	10.0	1.2
	2	20.0	2.4
Carp: No. 5, 6, 7*	4	20.0	0.8
	2	40.0	1.6
Fundulus: No. 9	4	19.9	70.0
	2	39.8	140.0
No. 10	4	19.5	28.0
	2	39.0	56.0
No. 11	4	19.7	20.0
	2	39.4	40.0
No. 12	2	19.8	24.6

* Each sample tested with same numbers of efts.

Fundulus (Lot 9): A total of 1700 glands weighing 199.5 mg. was prepared from freshly captured fish collected near New Haven, Connecticut, in October and November, 1957, plus a few from aquarium-kept fish of the same batch killed in December, 1957. All were in a state of complete sexual regression. The material was frozen in closed vials to prevent desiccation. The brei was prepared seven months later; homogenization was difficult, probably on account of the tough neural processes and diminished glandular material at this season of the year, but a smooth homogenate was finally prepared and diluted to 2 ml. in 0.6% NaCl.

(Lot 10): The greater part of the material (280 glands) was taken from pre-spawning fish captured near New Haven, Connecticut, *ca.* May 1, 1955 and kept up to ten days in the laboratory; 80 glands from freshly captured, early spawning fish caught June 7–8, 1955, were added to bring the total to the required weight. The material was stored for two years in closed vials in the deep freezer and the brei was prepared in the usual manner from a total of 360 glands weighing 195.5 mg.

(Lot 11): Two hundred glands weighing 197.5 mg. taken from freshly captured fish at the beginning of the spawning season, June 7–8, 1955, were prepared as described above.

(Lot 12): Three hundred and twenty glands weighing 129.0 mg. taken from freshly captured fish during the period of sexual regression in September and October, 1955 and kept frozen for two and one-half years in closed vials were homogenized in 1.3 ml. of 0.6% NaCl so that the concentration was equivalent to that used in the preceding samples.

In addition to the above material, tests were conducted on a lyophilized preparation (Wilhelmi, Lot F80x) derived from a mixture of hake, pollack and cod. The bulk of the glands were taken from hake (*Urophycis tenuis*) in post-spawning condition. The material was collected at Wilson's Beach, Campobello Island, New Brunswick, in the summer of 1952.

A number of efts collected near Petersham, Massachusetts in August and September, 1958 were hypophysectomized and kept for a period of two weeks before treatment. The animals varied in weight from 0.63 to 1.48 gm. and all were considered to be well removed from the naturally occurring water-drive phase of their life cycle. Each animal received intraperitoneal injections on each of two separate days of fish pituitary brei delivered at a standard volume of either 0.1 ml. or 0.2 ml. per injection. After the first injection the efts were placed in containers with a land and a water area and the time of their migration to water was noted. Changes in weight and length during a period of four weeks after the first injection were recorded in most instances. The data for the doses administered and the calculated number of pituitaries involved are given in Table I.

RESULTS

The results of the above experiments are summarized in Table II.

(a) Tests with pollack: Most animals tested with pollack pituitary failed to show the water-drive response. In all respects they appeared as non-treated hypophysectomized efts, their skin dark and dry as layers of cornified epithelium built up in the absence of normal molting factors presumably involving TSH. Two animals receiving brei from Lot 1 gave a partial response. Their skin sloughed off in rough patches and the efts entered water at irregular intervals, remaining there for several hours at a time. One animal, however, treated with Lot 4 material, gave a positive reaction by assuming the water habitat for a period of a week, during which time all of the old cornified epithelium sloughed off revealing the smooth, non-granular skin characteristic of the aquatic phase. Changes in weight and length were erratic but some individuals (8) undoubtedly grew slightly.

(b) Tests with carp: Fourteen out of the 18 individuals treated with carp pituitary gave a positive response on an average of about ten days following the initial injection and remained in water for periods of from two to three weeks. The four unresponsive individuals may be discounted. Two receiving Lot 5 brei failed to give even a partial response, but one of these was poorly injected so that some of the preparation was lost, while the second individual was suffering from an acute fungus infection. An additional two animals died shortly after the second injection, before any response could have been expected. The efts showed a mean increase in weight and length. All animals molted normally.

TABLE II

Results of water drive studies following treatment of efts with various teleost pituitary preparations

Donor material and lot numbers	No. test animals*	Wt. change per cent 4 weeks	Length change per cent 4 weeks	Results		Days to water average
				Positive	Negative	
Fundulus (post-spawning)						
Lot No. 9	6	not recorded		5 (1 dead)	0	9.2
Lot No. 12	2			2	0	8.0
Fundulus (chiefly pre-spawning)						
Lot No. 10	6	+7.41	+6.35	6	0	7.6
Fundulus (early spawning)						
Lot No. 11	6	not recorded		0	5	
Pollack (pre-spawning)						
Lot No. 1	6	loss and gain		2 (partial)	4	
Lot No. 2	6	loss and gain		0	6	
Lot No. 3	6	loss and gain		0	6	
Lot No. 4	6	loss and gain		1	5	8.0
Carp (late spawning)						
Lot No. 5	6	+8.41	+4.46	4	2†	12.0
Lot No. 6	6			5 (1 dead)		7.2
Lot No. 7	6	+8.70	+3.20	5 (1 dead)		12.6
F80x Wilhelmi—lyophilized hake, pollack, cod	6	not recorded		0	6	
Controls	16	−5.77	none	0	16	

* See Table I.

† See text.

(c) Tests with *Fundulus:* Injection of pituitary brei from fish in sexual regression (Lots 9 and 12) yield positive results in all animals tested, with the exception of one which died shortly after the first injection. The animals went to water between eight and nine days after their first treatment and molted normally. It is interesting to note that Lot 10, largely prepared from laboratory-kept animals in the early pre-spawning condition, also gave positive results, while tests with Lot 11 brei from spawning animals were entirely negative. Efts receiving Lot 10 increased significantly in weight and length; the other preparations were not studied in respect to the growth response. Animals receiving Lot 11 molted regularly, indicating that the preparation contained at least some endocrine activity.

Injections of the lyophilized preparation F80x gave negative results in all animals tested.

Controls remained in the terrestrial phase, did not molt, lost weight and showed no change in length.

CONCLUSIONS

These tests present convincing evidence that a prolactin-like substance is a naturally occurring factor in the pituitary glands of teleost fishes. Carp brei (Lots 5, 6, 7) gave a positive response as did material prepared from *Fundulus* (Lots 9, 10, 12) in the pre- and post-spawning conditions. The nature of this response indicates that this material, administered in doses of 10 or 20 mg., contained lactogenic hormone well above the estimated threshold of 1.4 μg. for initiation of the water-drive response. That there was no apparent difference in the nature and time of response between animals receiving 10 and 20 mg. of brei agrees with Grant (unpublished data) who has shown that at or above the 3 μg. level total response can be expected in all efts treated, provided they are in a healthy condition.

One of the animals receiving pollack preparations showed positive water-drive, while two others gave a partial reaction. Although the response was very weak in this case, the presence of prolactin in small amounts is indicated. This is in agreement with the work of Lehrman (in Pickford and Atz, 1957) who obtained positive results with pollack brei on the pigeon crop test. Both the present series of tests and those of Lehrman's gave negative results with the lyophilized powder F80x.

The fact that pituitaries taken from animals near the end of the spawning season and from those in the post-spawning condition, gave the strongest response is interesting. Lot 11 brei from early spawning killifish gave negative results while pollack brei from pre-spawning animals produced a very weak response. In the latter case the possibility of deterioration of the sample, which was partially dehydrated after four years' storage in the deep freezer, cannot be excluded, but the glands taken from *Fundulus* in June, 1958 are believed to have been in perfect condition. Further experiments are needed to exclude a possible unexplained deterioration of the sample. These tests may indicate a seasonal depletion in prolactin associated with the reproductive cycle, but the results are not decisive. Negative results with the lyophilized powder, chiefly from post-spawning hake, conflict with this hypothesis.

SUMMARY

It has been demonstrated, by means of the red eft water-drive test, that a prolactin-like hormone is present in the hypophysis of teleostean fishes. Pituitary

extracts from late spawning carp (*Cyprinus carpio*) and pre- or post-spawning killifish (*Fundulus heteroclitus*) gave a positive response in all instances. Pollack pituitary brei (*Pollachius virens*) from pre-spawning fishes gave only a weak response, and wholly negative results were obtained with an extract of *Fundulus* pituitary glands from fish taken at the beginning of the spawning season. While these data suggest a possible period of depletion during the early spawning phase of the sexual cycle, the findings require confirmation. The experiments also demonstrated that the pituitary of the three species investigated contains a growth-promoting factor and a molting hormone, presumably somatotropin and thyrotropin, respectively.

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DEVELOPMENTAL CHARACTERISTICS OF LOW TEMPERATURE CHICK BLASTODERMS. I. INFLUENCE OF THE HYPOBLAST ON DEVELOPMENT IN VITRO[1]

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This is the third paper dealing with investigations of the developmental characteristics of chick blastoderms incubated *in ovo* for extended periods at 25° C. Previous papers (Harrison and Klein, 1954; Harrison, 1957) reported on the formation and dispersal of the primitive streak during low temperature incubation, and on a differential sensitivity between neurogenesis and heart formation to low temperature incubation.

Depending upon the length of exposure to the low temperature, morphologically different blastoderms occur. The primitive streak forms but subsequently disperses in a posterio-anterior direction. Blastoderms containing partial streaks are called the "diffuse streak" type. As dispersal progresses blastoderms retaining only the node result. These are called "node" blastoderms. With continued incubation dispersal of the node occurs to yield the "diffuse node" type and finally blastoderms are formed which are devoid of axiation, the "anidian-like" blastoderms.

Interest in the present work resulted from a discrepancy in previous data (Harrison, 1957) which reported the adaptation of *in vitro* culture methods to the study of the low temperature blastoderms. Following incubation at 25° C. for 10 to 14 days, the blastoderms were removed, transected and cultured on a yolk-albumen medium. The percentage of explants developing normally after 10 days exposure was lower than that for explants exposed for 11 days. The sigmoid curve was not expected and did not agree with data published in 1954 (Harrison and Klein). A possible explanation seemed to be a variation in technique as the experiments progressed. The original method of transecting blastoderms was that followed by Spratt (1947b) in which he cut blastoderms approximately 0.2 mm. behind the primitive pit. This procedure was followed in the early experiments but very soon the level of the cut was shifted posteriorly near the junction of the area pellucida and area opaca. The developmental capacity of explants which retained more of the primitive streak area appeared to be better at that time and the procedure was subsequently followed in the experiments.

The present work reports on the attempt to determine if there is a significant difference in developmental potency of blastoderms transected at different levels. Due to the higher developmental potency of the diffuse streak blastoderms as opposed to the other degenerate types, this form was used. Since transection re-

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moves both epiblast and hypoblast, data are presented to show the effect of the removal of posterior hypoblast.

METHODS AND RESULTS

General

Fertile eggs were obtained locally from a two-year-old flock of White Rock chickens during the period June, 1956 through October, 1957. A total of 567 eggs was used and showed a fertility rate of 83%. All eggs were less than 24 hours old when placed in incubators, to minimize effects from storage. Incubation was carried out in two identical forced-draft, thermostatically controlled incubators having a relative humidity of 75 ± 5.0%. For low temperature incubation, the incubator was kept in a walk-in cold room with a temperature of 18° C., which permitted maintenance of an incubation temperature of 25° ± 0.5° C. The control incubator was maintained at the normal incubation temperature of 37.5° ± 0.5° C.

In all instances the period of low-temperature incubation was 9 days. On the basis of previous work (Harrison and Klein, 1954; Harrison, 1957), this particular period was selected as being one which would yield a high percentage of blastoderms containing diffuse primitive streaks. Of 390 fertile eggs which were incubated for 9 days at 25° C., 238 (61%) contained blastoderms with the diffuse streak.

The Petri-dish technique of tissue culture developed by Fell and Robinson (1929) and subsequently modified by Spratt (1947a) was used and is fully described elsewhere (Spratt, 1947b; Harrison, 1957). For the culture medium a yolk-albumen-saline-agar mixture was used. This was prepared under sterile conditions by adding the contents of one unincubated egg to 100 cm.^{3} of Ringer solution.[3] This mixture was shaken thoroughly and decanted to centrifuge tubes in such a manner as to leave the larger masses of albumen behind. The fluid was centrifuged for one hour at 825 g. Twenty cubic centimeters of the yolk-albumen supernatant were added to a sterilized mixture of 20 cm.^{3} of Ringer solution, 1 cm.^{3} of indicator solution (0.02 gm. phenol red/100 cm.^{3} solution), and 200 mgm. of Bacto Agar which had been cooled to 30°–40° C. After mixing, CO_2 was added by blowing through a sterile pipette with a cotton plug. One cubic centimeter of the medium was pipetted into each culture watch crystal.[4] The pH of the final medium varied from 7.4 to 8.1. Appropriate explants were placed on this medium and incubated at 37.5° C. for two days. At the termination of incubation, the morphology and degree of development of the blastoderms were recorded. They were then fixed in Bouin's solution. All explants were sectioned, stained with Delafield's hematoxylin, and counterstained with eosin.

In ovo controls

Two types of *in ovo* controls were used to determine the developmental potency of the intact blastoderms as opposed to those removed from the yolk. In the first type, 21 eggs were incubated for two days at 37.5° C. Of these, 16 were fertile and 15 or 93.8% produced normal embryos. From this particular flock of chickens, fertile eggs develop normally for the first two days in better than 90% of the cases.

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[3] NaCl, 0.9; KCl, 0.042; $CaCl_2$, 0.024 gm. per 100 cm.^{3} solution.

[4] The yolk-albumen medium was prepared fresh each morning of the day in which explantations were made. Storage of culture plates reduced the success of explantations.

In the second type 107 fertile eggs were incubated for 9 days at 25° C. followed by incubation for two days at 37.5° C. Fifty-one of these (47.7%) produced normal embryos. This potency for normal development was not as high as previously reported (Harrison and Klein, 1954). In the earlier work one group of 10 eggs was pretreated for 10 days at 25° C. Ninety per cent of the eggs produced normal embryos. In the present work, 11 groups of eggs were set, and the highest percentage of normal development for any group was 83.3% and the lowest was 0.0% normal development. The average of 47.7% is comparable to that obtained in 1957 (Harrison) and is more indicative of the true developmental potency of eggs exposed at 25° C. and subsequently incubated at 37.5° C. *in ovo*. The present control groups consisted of 5 to 26 eggs and represent a much better sampling than did the 1954 group. Differences between the groups, in terms of susceptibility to the low temperature exposure, seem to be the result of individual variations in eggs.

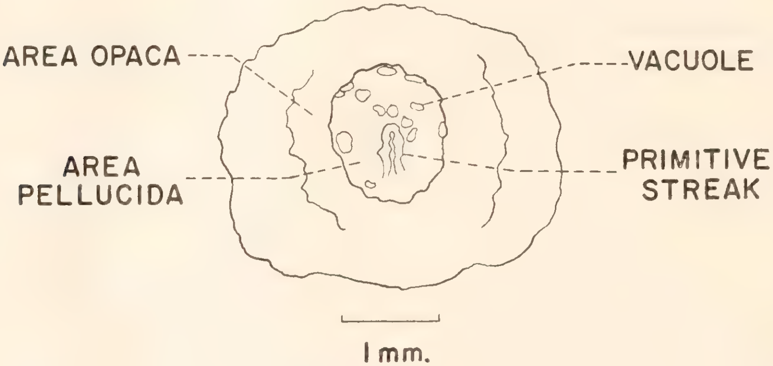

FIGURE 1. Morphology of a typical diffuse streak blastoderm resulting from incubation for 9 days at 25° C.

Variations in level of blastoderm transection

To determine whether or not the amount of streak which remained in a transected blastoderm affected its *in vitro* development, a series of experiments was performed transecting the blastoderm at different levels of the primitive streak behind the node. In all instances the blastoderms used were those previously classified as "diffuse streak." Figure 1 represents a typical diffuse streak blastoderm. In general, the area pellucida tends to be elliptical rather than narrow at the posterior portion as in normal definitive streak blastoderms. The posterior portions of the streak are no longer visible, indicating a posterio-anterior gradient of streak dispersal. The anterior end of the primitive streak remains but does not contain a clearly defined primitive pit area. Vacuolation has occurred in the area pellucida and frequently is more pronounced than shown in the figure. However, the degree of vacuolation is not correlated with ability to continue normal development.

In preparation for explantation, blastoderms were removed from eggs exposed to the low temperature. The morphology was determined and diffuse streak area was measured from the anterior rim of the primitive node posteriorly to the edge of the area pellucida with an ocular micrometer. A transverse cut was made at the desired level with two finely ground steel needles. Excess area opaca was trimmed from the anterior piece following the method of Spratt (1947b). The length of the streak in the anterior piece was re-measured, and it was then placed on the culture medium using a wide-mouth medicine dropper. Excess Ringer solution was removed with a fine pipette.

TABLE I

Summary of results with transected diffuse streak blastoderms incubated in vitro for two days

Expt. no.		*In vitro* development of the explants													
		Explants containing from node to 49% of the primitive streak							Explants containing from 50% to 100% of the primitive streak						
	No. explants	Normal	Abnormal	No development	Normal brain	Normal neural cord	Normal heart	Normal vitelline vessels	Normal	Abnormal	No development	Normal brain	Normal neural cord	Normal heart	Normal vitelline vessels
40	4	0	0	4	0	0	0	0							
58	8	1	1	6	1	1	1	2							
56	8	0	1	2	0	0	1	1	2	3	0	2	2	4	5
51	10	0	4	4	0	1	2	4	1	0	1	1	1	1	1
36	7	1	0	1	1	1	1	1	0	3	2	0	1	2	2
34	9	0	4	1	0	0	2	4	2	1	1	2	3	2	3
55	7								4	2	1	4	5	5	6
35	9								1	4	4	1	2	3	3
31	9								3	6	0	3	3	4	7
33	10								4	1	5	4	5	4	4
37	6								2	3	1	2	2	3	4
32	10								2	6	2	2	2	4	7
39	9								2	6	1	2	4	3	7
Totals 13	106	2	10	18					23	35	18				

Table I summarizes the results of these explants after two days of incubation at 37.5° C. One hundred and six diffuse streak blastoderms were transected and the anterior portions explanted to the yolk-albumen medium. Thirty of the explants included only the primitive node region or up to 49% of the primitive streak. Seventy-six of the explants retained from 50% to 100% of the primitive streak tissue. Of the explants retaining 49% or less of the primitive streak tissue only 6.7% developed normally and 60% showed no development during the period of incubation. The remaining 33.3% were classified as abnormal embryos. These are embryos which are normal in at least one morphological system, *i.e.*, nervous, circulatory (heart *and/or* vitelline vessels), closed gut or notochord, as revealed by histological examination of serial sections. As opposed to these, explants which had no discernible embryonic systems or which lacked at least one normal system were classified as "no development."

In contrast, explants which retained from 50% to 100% of the streak tissue developed normally in 30.3% of the cases. Forty-six per cent of the explants were classified as abnormal and 23.7% showed no development. This developmental difference proved to be significant when a coefficient of correlation was made for variation between individual experiments as opposed to occurrence of developmental types. Comparison of normals, abnormals and those showing no development for explants with 49% or less of the primitive streak gave an F-value significant at the 0.1% level ($F_{20.45}$; $n_1 = 2$, $n_2 = 15$). The same comparison for explants retaining 50% to 100% of the primitive streak was not significant. Differences in the percentages of the developmental types were a factor of chance rather than the result of transecting the embryo. The developmental potency of transected embryos is enhanced when the level of transection is such that the explants retain more than 50% of the primitive streak tissue.

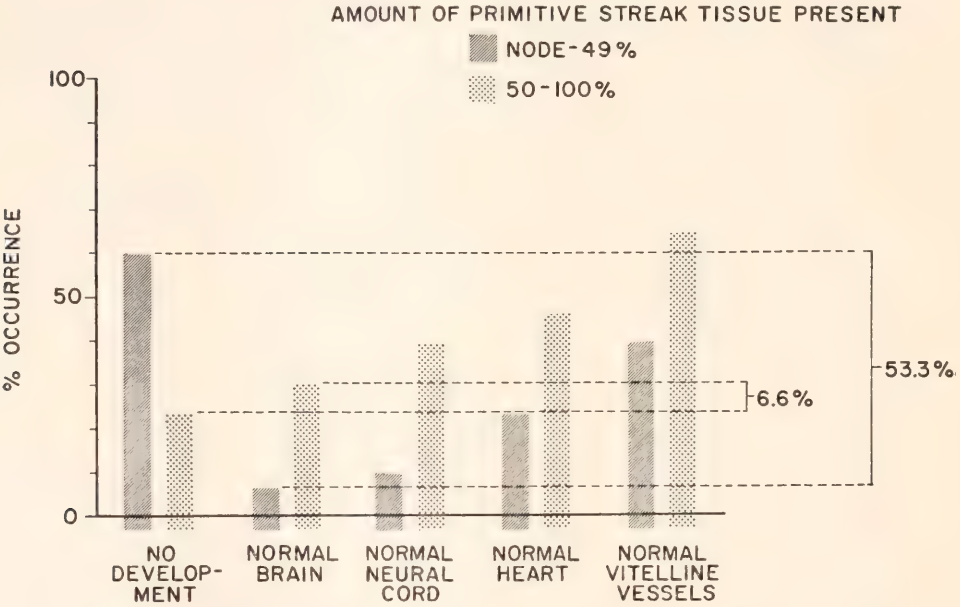

FIGURE 2. Comparison of explant development in relation to amount of primitive streak tissue present.

Figure 2 summarizes material contained in Table I. The trend in developmental types shown by explants containing 49% or less of the primitive streak tissue is significant at the 1% level ($F_{5.066}$; $n_1 = 4$, $n_2 = 25$) and the trend for explants with 50–100% of the primitive streak is significant at the 0.1% level ($F_{7.798}$; $n_1 = 4$, $n_2 = 50$). Explants which possess a normal brain are normal with respect to all other systems. Consequently the percentage given is the same for those classified normal. The difference between this percentage and the percentage of explants showing no development is 6.6% for explants containing 50–100% of the primitive streak. For explants retaining 49% or less of the primitive streak, the same percentage difference is 53.3%. This striking contrast in developmental potency between the two types of explants is significant, since both of the over-all trends are significant, and illustrates the necessity of including posterior portions of the primitive streak in explants.

Removal of posterior hypoblast

Establishment of the importance of the posterior portion of the primitive streak region for development of explants posed the problem as to whether or not the hypoblast of this region was the stimulating tissue. In an attempt to answer this the following experiments were performed. Low-temperature blastoderms were removed from eggs and diffuse streak blastoderms were selected for explantation. The most successful operative steps were as follows. Excess area opaca was removed from around the entire area pellucida. The length of the primitive streak tissue was measured from the anterior edge of the primitive node posteriorly to the edge of the area pellucida. The blastoderm was then inverted and the measurement checked from the ventral surface. With finely ground steel needles the hypoblast was cut at right angles to the streak axis approximately at a level which would leave 37% of the primitive streak tissue anterior to the cut. This is shown in Figure 3 by the anterior margin of the stippled area. This transverse cut was continued posteriorly and parallel to the primitive streak axis at each edge to the junction of the areas pellucida and opaca. This flap of hypoblast was peeled back by gently teasing with the needles. Removal of the hypoblast was most difficult

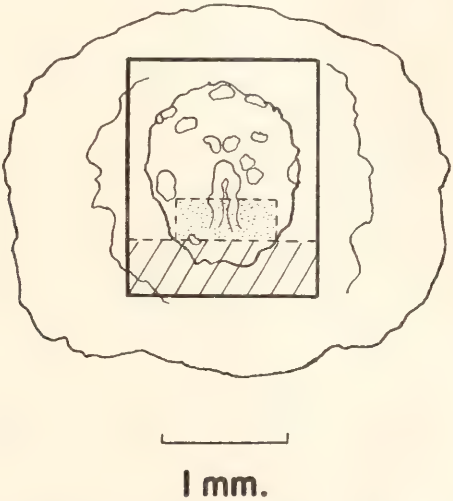

FIGURE 3. Preparation of diffuse streak blastoderm for explantation in hypoblast removal experiments. See text for discussion.

in the streak region where it had an intimate fusion with the tissue of the epiblast. The operation was completed by making a transverse cut across the entire tissue at a level which left approximately 75% of the primitive streak epiblast intact. The explant was transferred to the culture medium.

Figure 3 summarizes schematically the steps involved in preparing a blastoderm for explantation. The solid line indicates the method of trimming the blastoderm to remove the excess area opaca. The stippled area represents the portion of the hypoblast that is removed. The cross-hatched area is discarded after transection, leaving approximately 75% of the primitive streak tissue in the anterior blastoderm portion to be explanted. Essentially the explant retains 75% of the original streak area from which about half of the underlying hypoblast is removed to leave approximately 37% of the original hypoblast intact.

Table II

Summary of results in the hypoblast removal experiments

Expt. no.	No. explants	*In vitro* development of explants during two days of incubation at 37.5° C.													
		Explants with hypoblast removed							Explants with hypoblast intact: Controls						
		Normal	Abnormal	No development	Normal brain	Normal neural cord	Normal heart	Normal vitelline vessels	Normal	Abnormal	No development	Normal brain	Normal neural cord	Normal heart	Normal vitelline vessels
82	12								4	6	2	4	6	9	10
83	10	2	2	0	2	2	3	4	3	1	2	3	4	2	5
84	12	4	1	0	4	5	5	5	4	3	0	4	6	7	7
85	9	2	3	0	2	2	5	5	4	0	0	4	4	4	4
89	3								1	1	1	1	1	1	1
90	8	2	1	1	2	2	3	3	2	1	1	2	2	3	3
91	9	0	4	1	0	2	4	4	1	3	0	1	2	4	4
92	8	1	1	2	1	1	2	2	1	1	2	1	1	1	2
93	12	2	1	3	2	2	2	3	3	0	3	3	3	3	3
Totals 9	83	13	13	7					23	16	11				

The decision to transect the blastoderms at a level which would leave approximately 75% of the primitive streak area intact was based on the foregoing experiments dealing with level of cut. And in this present series of experiments a control group consisted of blastoderms with the excess area opaca removed and transected so that about 75% of the primitive streak area remained intact. The only difference was that the hypoblast was left intact.

Eighty-three explantations were made. In 33 of these hypoblast was removed, and the remaining 50 were controls. The nature of the development of these explants during two days of incubation at 37.5° C. is summarized in Table II. Examination of serial sections of explants from which the hypoblast was removed showed that in all instances the hypoblast reconstituted during the two day incubation period.

The information in Table II is summarized in Figure 4. The information in the graph was analyzed for coefficient of correlation to see if the trends in development types for both kinds of explant were significant. The trend as seen in the graph for development of explants with hypoblast removed is significant at the 0.1% level ($F_{7.121}$; $n_1 = 4$, $n_2 = 30$). The F-value for analysis of the trend in development of explants with hypoblast intact is significant at the 1% level ($F_{3.587}$; $n_1 = 4$, $n_2 = 40$). Removal of the hypoblast results in a decrease in developmental potency. Although there is a very slight (0.8%) difference in the numbers of embryos showing no development in the two types of explants, there is a significant lowering in the numbers of explants showing normality of the nervous system. The hypoblast of the posterior primitive streak region has some influence on the normal development of the central nervous system. In contrast, the development of the circulatory system, heart *and/or* vitelline vessels, is not affected. This specificity of hypoblast influence would be more clearly indicated if it were possible to show significance for the difference in percentage between explants possessing a normal heart as opposed to those with a normal brain for control explants in contrast to explants with hypoblast removed. However, a coefficient of correlation for this difference shows that there is as much variation between individual experiments as between explant types. This lack of significance can be explained on the basis of developmental sequence of the two systems. The heart forms later in development than does the brain. Explants which do not form a heart in the two days of *in vitro*

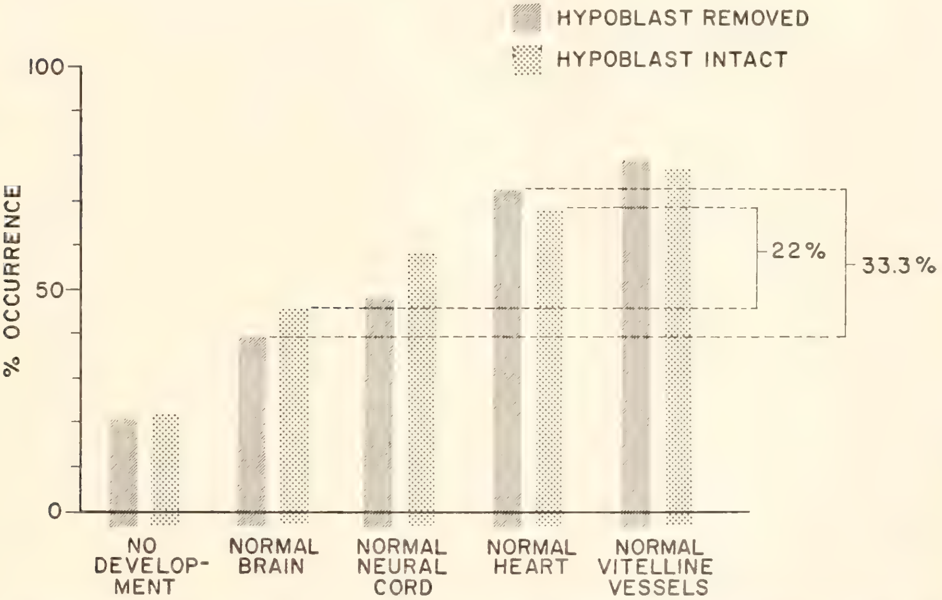

FIGURE 4. Comparison of explant development in relation to presence or absence of hypoblast.

incubation may be expressing a slower developmental rate rather than abnormal development.

Discussion

In any series of experiments which by necessity disrupt the normal conditions under which an embryo develops, it is important to determine the extent of the disruption. The various types of controls maintained in the present experiments provide an opportunity to determine if application of the *in vitro* culture technique has influenced the results. Of the series of *in ovo* controls which were exposed only to incubation at the normal 37.5° C. temperature for 2 days, 93.8% developed normally. In contrast, fertile eggs which were first incubated for 9 days at the lower temperature (25° C.) and then incubated *in ovo* at 37.5° C. contained embryos which developed normally only 47.7% of the time. This decrease in developmental potency can only be ascribed to the effect of low temperature exposure. The point of interest is that blastoderms which were exposed *in ovo* at 25° C. for 9 days and then cultured *in vitro* at 37.5° C. as transected blastoderms developed normally in 46% of the cases. This percentage is for explants under optimal conditions, *i.e.,* those containing 75% of the primitive streak material. The close correspondence of developmental capacity of low temperature blastoderms *in ovo* and *in vitro* indicates that application of the culture method is valid and does not affect the developmental potency of blastoderms in terms of the first two days of development.

The drop in developmental potency when posterior portions of the primitive streak region are removed by transection or dissection indicates that tissue of this region is concerned with normal development of the central nervous system, primarily with morphogenesis of the brain. Specifically those experiments involving removal of the hypoblast show that the tissue which is related to this process is the hypoblast. Transection at levels which remove the posterior half of the primitive streak region results in a drop in developmental potency which can be duplicated by removal of the hypoblast from that area while leaving the epiblast intact. This does not imply that the drop in developmental potency is reflected in a great increase in the numbers of explants which show no development. Rather the striking change is in the numbers of embryos that are abnormal as a result of mal-development of the central nervous system.

A corresponding influence, or organizer-like activity, of the posterior hypoblast is not shown in work with normal definitive streak blastoderms. Fraser (1954) investigated the role of the posterior hypoblast in normal primitive streak embryos and concluded that this tissue is not essential. The difference in conclusions may be explained on the basis that the low temperature blastoderm and the normal definitive streak blastoderm are not physiologically and developmentally identical. The ability of a diffuse streak blastoderm which has not undergone involution (Harrison, 1957) to re-form a primitive streak indicates that it still retains developmental capacities of a normal pre-streak blastoderm. It is conceivable that the posterior hypoblast of the pre-streak blastoderm possesses an organizer-like activity that has been expressed by the time the blastoderm attains the definitive streak stage. This concept agrees with Waddington's work (1933) in which he showed that pieces of posterior hypoblast were capable of inducing axiation in anterior epiblast which normally gives rise to extra-embryonic material.

If an organizer activity by the posterior hypoblast is assumed, it is possible to hypothesize that the effect of lowered temperature is upon the hypoblast. The greater sensitivity of the nervous system to low-temperature incubation as opposed to that of the heart *and/or* vitelline vessels would be an expression of hypoblast sensitivity. This would explain the apparent residual effect of low-temperature incubation, *i.e.,* low temperature exerts its effect at a time in development before the central nervous system begins to form.

Summary and Conclusions

1. This paper reports work showing the influence of posterior hypoblast on the *in vitro* development of diffuse streak blastoderms produced by incubation at low temperature (25° C.).

2. Normal *in ovo* controls incubated at 37.5° C. for two days show 93.8% normal development. *In ovo* controls incubated at 25° C. for 9 days followed by two days' incubation at 37.5° C. produced normal embryos in only 47.7% of the cases. Transected blastoderms explanted to a yolk-albumen medium following 9 days' exposure *in ovo* at 25° C. developed normally in 46% of the cases. Thus the drop in developmental potency is the result of low-temperature exposure rather than transection.

3. Fertile eggs were incubated for 9 days at 25° C. The blastoderms were removed from the yolk and only those containing a diffuse streak were chosen for transection and explantation to a yolk-albumen medium. The results show that explants which retain only the primitive node region or up to 49% of the primitive streak tissue have a lower developmental potency than explants in which 50–100% of the primitive streak region was retained.

4. A series of explants were transected in such a manner that approximately 75% of the original primitive streak epiblast and about 37% of the original hypoblast beneath the streak remained. In comparison with explants which retained 75% of both hypoblast and streak region, those with hypoblast removed show a significantly lower developmental potency with respect to formation of normal brain.

5. The present results are discussed in correlation with previous results dealing with the effect of low temperature incubation on morphogenetic processes.

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A STUDY OF THE POLYSACCHARIDE HISTOCHEMISTRY OF THE OVIDUCT OF THE NEWT, TRITURUS VIRIDESCENS[1]

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The physiological importance of the jelly secreted by the oviducts of amphibia has become increasingly apparent in recent years, especially so with regard to the process of fertilization (Good and Daniel, 1943; Bernstein, 1952; Kambara, 1953; Nadamitsu, 1953, 1957; Tchou-Su and Wang Yu-Lan, 1956; Hughes, 1957). Previous work in this laboratory seems to show that the jelly is somehow involved in the normal blockage of meiosis in the eggs of *Triturus viridescens* (Humphries, 1955, 1958, and unpublished); thus for our work, it had become almost essential to know more of the nature of the oviducal secretions and something of the parts of the oviduct which were actively involved.

The oviducts of *Triturus viridescens*, as well as those of other urodele amphibians, exhibit a gross and histological differentiation into rather well-defined regions (Adams, 1940, 1950; von Wahlert, 1953). Adams (1940) described six regions: (1) the infundibular region, (2) a transparent region with a watery secretion, (3) an opaque region with eosinophilic granules, (4) a transparent, wider section, similar to section 2, (5) an opaque, chalky-white region, similar to section 3, and (6) a straight vaginal section with fewer folds and fewer secretory cells, which leads to the cloaca. The first three lie anterior to the kidney, the remaining three lie parallel to the kidney. For our purpose, however, it seemed desirable to designate the divisions of the oviduct somewhat differently, as follows: an infundibular, or ostial region, apparently non-secreting, at the extreme anterior end; a region A, just posterior, of gray color, ending abruptly in a white, or B region, of about the same diameter, which gradually decreases in diameter and whiteness until it passes into region C, a division of small diameter and gray color. Region D begins abruptly as a much wider section of dull white coloration, and region E as a division with about the same diameter as D, but with a brighter white color. The posterior part of E is narrower and less convoluted than its anterior portion. Our region A seems to correspond to region 2 of Adams, B and C to her region 3, D to her region 4, and E apparently includes regions 5 and 6.

In view of the striking regional zonation of the oviduct and what is known of the chemistry of amphibian egg jelly, it appeared that an investigation of the polysaccharide histochemistry of the secretory portions might provide useful information.

MATERIALS AND METHODS

All animals were collected in April from the vicinity of Franklin, North Carolina, and maintained in a refrigerator at about 12° C. until used. Oviducts were,

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in general, taken from animals which had been treated with one or two injections of Antuitrin "S" (Parke, Davis) to induce ovulation, but several animals were taken directly from the refrigerator and immediately sacrificed without treatment. No differences between the oviducts of treated and untreated animals were observed. In either case, the oviducts were in the normal condition for animals in the breeding season. Oviducts were, in one instance, taken from an animal which had completed her breeding activity, and in this animal the oviducts were considerably smaller in diameter and less convoluted than in females in breeding condition. The results presented here represent studies of the oviducts of thirteen animals, though not all of these were used for all procedures.

Periodic Acid-Schiff Method: Portions of the oviduct were fixed in Bouin's fixative, 10% formalin, or Carnoy's fixative, embedded in Tissuemat, and sectioned at 10 microns. The PAS procedure was carried through on both untreated sections and sections treated with saliva or malt diastase for removal of glycogen.

Toluidine blue: Fixation was in Bouin's fixative, 10% formalin, Carnoy's fluid, or in 4% basic lead acetate followed by formalin. Sections were stained for ten minutes in a 0.03% solution of toluidine blue O in a citrate buffer at pH 3.8, after which they were dehydrated in alcohol, cleared in xylene, and mounted in Clarite or Canada balsam.

Alcian blue: Fixation was in Carnoy's fluid or 10% formalin. Most of the staining was done with Alcian Blue 8GX 300, kindly supplied by Arnold, Hoffman and Company, Providence, Rhode Island. However, several slides from each region were stained with alcian blue obtained from the Hartman Leddon Company. For our purposes the dye from the former source was considerably superior, since it appeared to have a greater differential specificity for the several regions of the oviduct. Sections were stained for twenty minutes in a 0.3% solution of the dye in 3% acetic acid (pH 2.5 to 2.6).

Hyaluronidase: Nearly all the tests with hyaluronidase involved material from region A only. Testicular hyaluronidase, assayed at approximately 300 U. S. P. units per mg., was obtained from Nutritional Biochemicals Corporation. Sections were treated in solutions of enzyme in 0.3% NaCl at pH 5.7 or in a phosphate buffer at pH 6.7, at 37° C. Several concentrations and periods of treatment were used, ranging up to 60 U. S. P. units per ml. for as long as eighteen hours. Staining was done with toluidine blue, as described above. Controls consisted of alternate sections placed in comparable solutions of boiled enzyme and in enzyme-free solutions.

Ribonuclease: Ribonuclease (Worthington) was used in a 0.1% solution at pH 5.9. Sections (chiefly from region A) were treated for periods of one hour or six hours at 37° C. Following treatment, sections were stained for ten minutes in toluidine blue, as described above, in 1% pyronin Y in aqueous solution at pH 3.4, or in 1% pyronin Y in a phosphate buffer at pH 6.7.

OBSERVATIONS

All the descriptions to follow refer to the secretory epithelium alone. In general, regions A, B, and D showed reactions in common while regions C and E responded differently but like each other.

The entire secretory portion of the oviduct stained intensely with the PAS technique. The ostial region, which presumably is relatively non-secretory, and is characterized by very low epithelium, stained pink. The A region stained a bright purplish red, whereas regions B, C, D, and E stained a distinctly different shade which might be called reddish purple. The staining of all portions remained unchanged after treatment with saliva or malt diastase.

There was a marked difference in the response of the several regions to toluidine blue. The staining of the ostial region was orthochromatic. Region A showed violet to purple metachromasia in all cases. This region in the one post-breeding animal also showed metachromasia after all fixatives; however, the color produced in lead acetate-fixed material was chiefly blue. The metachromasia of region A is alcohol-fast. After destaining for periods up to one and one-half hours in 70% alcohol, the major part of the stain was removed, yet the violet metachromasia persisted. Region B usually stained an orthochromatic dark blue, but in some cases strong tinges of purple were seen. There was, however, no difficulty in distinguishing between regions A and B following this stain. Sections from region C showed little or no cytoplasmic stain, and that which was present was usually a very light blue. In a few cases there were traces of metachromasia. The staining reaction of region C, then, was a striking contrast to that of either A or B. Preparations from region D were variable in their response to toluidine blue, but, for the most part, the cytoplasm stained a pale blue, and nearly all preparations showed at least some violet metachromasia. The cytoplasm in cells of region E stained a pale blue, with no trace of metachromasia.

The reaction of the several regions to alcian blue was also varied. The cytoplasm of the secretory cells of region A stained a bright sky blue, as did that of region B. The cells of region C, however, stained only lightly. In many cases the stain in this region was so light as to be only barely detectable. The cytoplasm of the cells of region D stained sky blue, similar to the reaction of regions A and B, while that of region E was extremely lightly stained, similar to the reaction of the cells of region C.

Although only one slide from each region was used, it may be worthwhile to mention that results with Hale's technique were essentially the same as those with alcian blue, but gave promise of less clear differentiation between the regions.

Treatment with hyaluronidase failed to alter the strong violet metachromasia of region A, or, in the few tests run, the staining reactions of regions B, C, or D. Similarly, treatment with ribonuclease failed to alter the staining reaction of the cytoplasm of the cells of either region A or region B. No other regions were tested.

Discussion

These results show the secreting epithelium of the newt oviduct to be rich in polysaccharide, apparently distributed qualitatively among the several regions. The histochemical differences follow the zonation which is anatomically demonstrable. It is clear that the epithelium contains polysaccharide other than glycogen, since the PAS reaction of all regions remains unchanged after glycogen digestion. The PAS technique allows but little differentiation between the regions, however, since region A is the only one which stains in a fashion distinctly different from the others.

A striking parallelism between metachromatic staining with toluidine blue and a positive reaction with alcian blue has been previously shown (Vialli, 1951; Wagner and Shapiro, 1957). This finding was clearly borne out in our material. Unfortunately, the metachromatic staining reaction has had a long history of confusion as to application and interpretation, but "true" metachromasia, in the sense of Lison (1953) or Kramer and Windrum (1955), generally is taken to indicate the presence of sulphated mucopolysaccharides, though nucleoprotein has also been reported to stain metachromatically at times (Wiame, 1946; Penney and Balfour, 1949; Kramer and Windrum, 1955). The occurrence of alcohol-resistant beta (violet) metachromasia, such as encountered in our material, is strong presumptive evidence of nucleoprotein, according to Kramer and Windrum (1955). However, we found no change in staining reaction with toluidine blue or with pyronine following treatment with ribonuclease. Results of several workers (especially Vialli, 1951; Lison, 1954; Mowry, 1956; and Wagner and Shapiro, 1957) indicate that alcian blue positivity is good evidence for the presence of acidic carbohydrates. Combining the evidence, then, the distinctive staining reactions of region A, in particular, and probably those of regions B and D, would seem most likely attributable to the presence of acid polysaccharide. The results with hyaluronidase seem to rule out the possibility that the distinctive reactions, at least of region A, are due to hyaluronic acid or anything very closely related to it.

Similar findings with respect to the PAS reaction, toluidine blue metachromasia, and hyaluronidase treatment have been reported for the Japanese newt, *Triturus pyrrhogaster*, by Kambara (1956a, 1956b, 1957a, 1957b). Due to lack of certainty as to corresponding regions in oviducts of the two species, it is not possible to make more than a rough comparison of our results with those of Kambara, but our results show strong general agreement with his.

The most probable conclusion to be drawn, at present, from the findings of distinctive reactions along the oviduct is that the differential staining is due to the presence of acid polysaccharide in regions A, B, and D only, with the positive PAS reaction of regions C and E due perhaps to neutral polysaccharide. However, quantitative differences alone might account for the results. Since PAS positivity and strong metachromasia do not tend to coexist, it is worthwhile to note the suggestion of Hale (1957), that the combination may be caused by the presence of two distinct substances. Our evidence, coupled with evidence from studies on the chemistry and physiology of egg jellies themselves (Immers and Vasseur, 1949; Vasseur, 1952; Kelly, 1954; Minganti, 1955; Runnström and Immers, 1956), leads to the conclusion that the metachromasia and alcian blue positivity of regions A, B, and D are probably due to the presence of a heparin-like compound. If this is the case, and if, as seems quite probable, the innermost layer secreted about the egg contains the substance, it may very likely have significant effects upon the physiology of the maturing oocyte. The effects of heparin and heparin-like compounds on the physiology of cells, especially egg cells, have been studied for years, particularly by Heilbrunn and his co-workers (see Heilbrunn, 1956, for references). In addition, one of us (Humphries, 1955, 1958) has obtained results which have led to the hypothesis that the oviducal jelly plays an important role in the natural blockage of the second meiotic division in the oocyte prior to fertilization. Oocytes never exposed to oviducal jelly, such as coelomic eggs and eggs stopped experimentally in the ostial (non-secreting) part of the oviduct, are capable of completing meiosis, while eggs exposed to jelly have in no case been seen to advance beyond the normal stage of blockage, metaphase II. It is perhaps significant that as the oocyte enters the first secreting portion of the oviduct it is completing the first meiotic division or beginning the second (Humphries, 1956). One of the possible explanations of the meiotic blockage is that the oviduct secretes an antimitotic substance. This possibility led to the present study of the histochemistry of the oviduct, with the aim of gaining information concerning the secretions of particularly the more anterior regions. Since heparin-like substances have been shown to act as antimitotics (see especially Heilbrunn, 1956, and Heilbrunn *et al.,* 1957), the discovery that oviducal region A apparently produces a heparin-like compound is in good agreement with the hypothesis. If this type of antimitotic is involved, however, it is surprising that blockage of the division occurs at metaphase, rather than prior to spindle formation.

SUMMARY

1. Application of some techniques of polysaccharide histochemistry to the oviduct of the newt showed a histochemical differentiation of the secretory epithelium corresponding to the grossly and histologically demonstrable zonation of the oviduct. All regions responded positively to the PAS technique. No difference was detected in sections previously exposed to glycogen digestion methods. Regions designated A, B, and D were metachromatic with toluidine blue and reacted positively to alcian blue.

2. The most probable explanation of the differences in staining reaction seems to be the presence of an acid polysaccharide, probably a heparin-like compound, in regions A, B, and D, and its absence (or much lower concentration) in regions C and E.

3. The possible significance of the findings relative to the physiology of the oocyte, particularly with regard to meiotic blockage, is discussed.

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THE CHEMICAL CONTROL OF FEEDING IN THE PORTUGUESE MAN-OF-WAR, PHYSALIA PHYSALIS L. AND ITS BEARING ON THE EVOLUTION OF THE CNIDARIA[1]

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The remarkable demonstration by Loomis (1955) that reduced glutathione (GSH) induces a feeding response in *Hydra* led us to examine the feeding responses of other cnidarians. Our object was to see whether the chemical control of feeding occurred in other hydrozoans and to identify the chemicals involved. The first organism selected was the Portuguese man-of-war, *Physalia physalis* L. Many different types of zooids suspend from the crested pneumatophore, or float, of this colonial hydrozoan (Figs. 1–4). Among the most numerous are the gastrozooids (Fig. 5), which are the only members of the colony capable of ingesting food. These gastrozooids have the usual polyp form, but lack tentacles. In the feeding process, the prey is drawn up to the gastrozooids which apply their mouths to the surface of the prey; the lips of the gastrozooids then spread out until they envelop the prey (Fig. 4) and digestion proceeds. This process has been described briefly by Wilson (1947). The present report describes the chemical control by GSH of the behavior of both isolated and attached gastrozooids, and discusses the possible evolutionary significance of these findings.

MATERIALS AND METHODS

Physalia were captured off the coast of Woods Hole, Massachusetts, in the summers of 1957 and 1958 and were maintained in aquaria supplied with fresh running sea water. A total of fifteen colonies were studied. *Hydra littoralis* were grown by the methods of Loomis and Lenhoff (1956).

Experiments testing the effects of GSH and cysteine were performed in the following manner:

1) Groups of gastrozooids were removed from the float with forceps and scissors. Single polyps were separated, collected in a finger bowl, and rinsed several times to remove any fluids that had oozed from the cut surface. This washing prevented most of the spontaneous opening of the gastrozooid mouth which occasionally occurred after the gastrozooids were isolated. Apparently this spontaneous mouth opening is a response to some substance released from either the cut surface of the gastrozooid itself, or from recently ingested food in the cavity of the gastrozooid.

2) The rinsed gastrozooids were distributed randomly in a series of finger bowls, each containing 90 cc. of fresh sea water (non-aerated). Only newly isolated gastrozooids with closed mouths were used.

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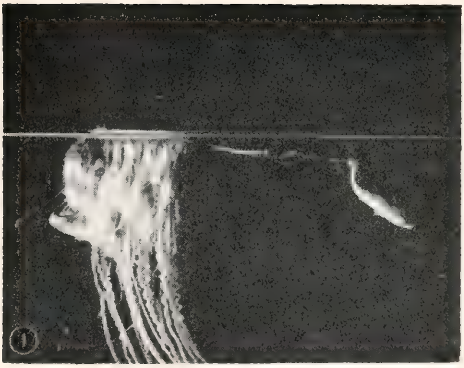
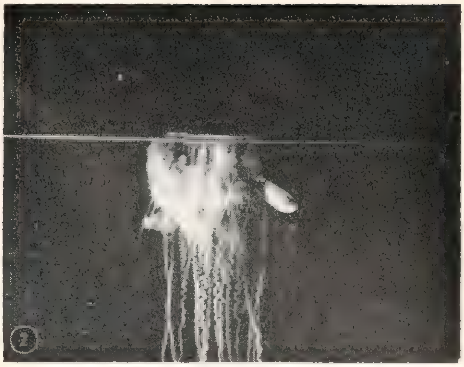
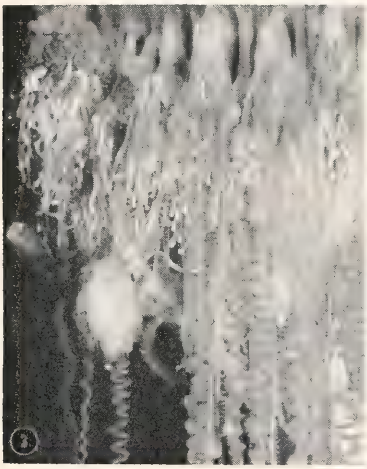






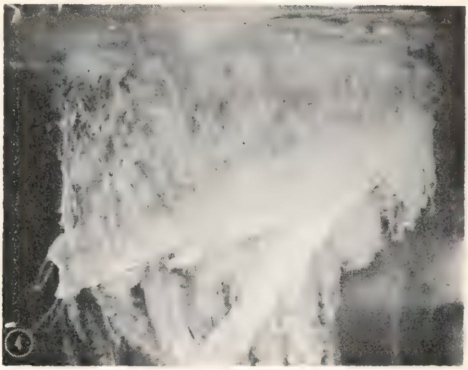


FIGURE 1. *Physalia* fishing tentacle captures small fish.

FIGURE 2. Fishing tentacle draws fish into colony of zooids.

FIGURE 3. Enlargement of *Physalia* zooids. Note the long coiled fishing tentacle, and the many small finger-like, white tipped gastrozooids.

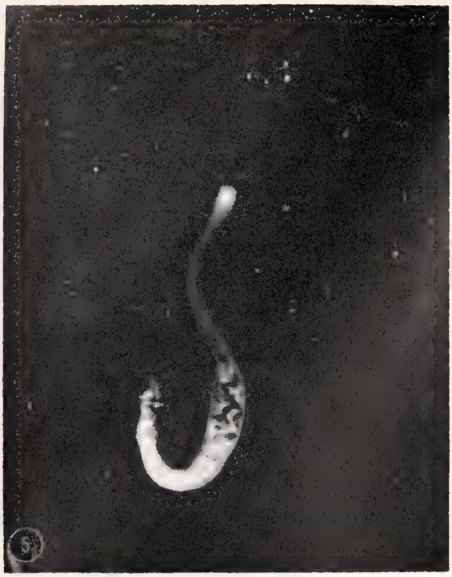
FIGURE 4. Gastrozooids envelop captured fish. Note, in the area of the caudal fin, the mosaic pattern formed by the rims of the spreading gastrozooids in contact with each other. Compare this arrangement with that of the isolated spreading gastrozooids shown in Figure 7.

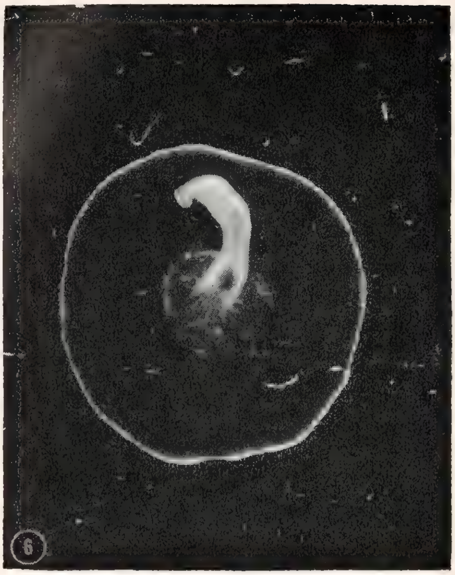
3) After the gastrozooids were added to the finger bowls, 10 ml. of a freshly-prepared, neutralized solution of GSH or cysteine were added to give final concentrations ranging from 10^{-3} to 10^{-8} M.

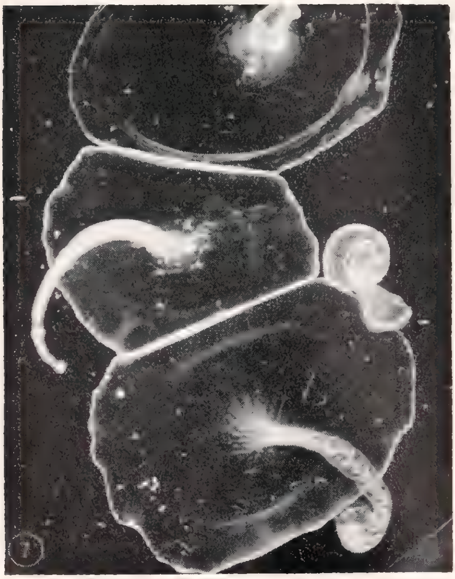
EXPERIMENTAL RESULTS

1. *Observations of the "feeding response" of Physalia gastrozooids*

In the experiments to be described, the gastrozooids exhibited a specific "feeding response," which is described here in detail. Several of the stages are illustrated in Figures 5–8.







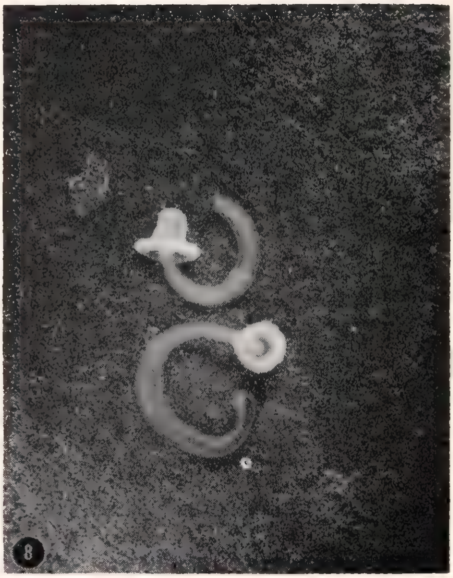


FIGURE 5. Isolated gastrozooid. The mouth is at the uppermost part at the end of the narrow cylindrical neck.

FIGURE 6. Gastrozooid induced to spread by GSH. Note the many small batteries of nematocysts along the rim of the gastrozooid lip.

FIGURE 7. Several GSH-induced spreading gastrozooids cover a large surface area.

FIGURE 8. Gastrozooids turned partially inside out by GSH.

Upon addition of GSH or fresh fish blood, the isolated gastrozooids immediately began to writhe. Within five minutes their mouths opened. Whenever a mouth contacted a solid object, such as a fish or a glass surface, it attached itself and began spreading (Fig. 6) as if to enclose the object in conjunction with the other spreading gastrozooids (Fig. 7). The gastrozooids, normally about 1 to 2 mm. in diameter, frequently spread to a diameter of 20–25 mm. By this process many small gastrozooids could surround and digest a large fish (Fig. 4). The spreading phenomenon usually took about one-half hour from initial contact with the solid object until the maximum diameter was reached. This spreading of the gastrozooids may persist for only a few minutes or last for two or more hours. The duration probably depends in part upon the nature of the surface to which the gastrozooid is attached (*i.e.,* smoothness, etc.) and upon their nutritional state. Occasionally spreading gastrozooids detached from the solid object and folded back over themselves, sometimes completely inverting so that endoderm was on the outside and ectoderm on the inside (Fig. 8). A similar phenomenon was described by Loomis (1955) in *Hydra.*

Some outspread gastrozooids migrated slowly over the surface of the solid object leaving a "mucous" trail. The polyp probably secreted extracellular proteolytic enzyme in this mucus to partially break down its prey.

2. *Feeding response of the intact animal*

A small piece of filter paper, soaked in a 10^{-3} M solution of GSH, was placed several centimeters from the gastrozooids of an intact *Physalia.* A typical feeding response occurred, with active squirming of the gastrozooids and then a spreading of their mouths on the paper and on the wall of the aquarium.

3. *Demonstration of a chemical feeding mechanism using live prey*

Ten gastrozooids and a small killifish (*Fundulus* sp.) (8 cm.) were placed in a finger bowl. After a half hour no feeding response of the gastrozooids was observed. At this time a fragment of a fishing tentacle armed with a large number of nematocysts (Fig. 3) was dropped onto the fish. The fish thrashed about for a moment and became immobilized. Within 10 to 15 minutes most of the gastrozooids began spreading on the surface of the finger bowl, apparently in response to some substance released from the pierced fish. Had the gastrozooids been close to the fish (as occurs when the intact colony draws the prey up to the float) then doubtless their mouths would have enveloped the fish (Fig. 4).

4. *The effect of different concentrations of reduced glutathione*

Ten gastrozooids were placed in several concentrations of GSH. The number of gastrozooids spreading was counted at intervals. As shown in Table I, after approximately two and one-half hours, 80–90% of the gastrozooids in $10^{-5}-10^{-6}$ M GSH had spread their mouths over the surface of the glass bowl. The fact that higher concentrations failed to elicit this response is not unusual, and finds a parallel in *Hydra* where concentrations of GSH above optimum caused a tight closing of the animal's mouth. Also, as in *Hydra,* concentrations of GSH less than 10^{-7} M were ineffective.

TABLE I

*Number of gastrozooids spreading in different concentrations of reduced glutathione after various time intervals**

Time (min.)	10	35	70	105	155
Reduced glutathione concentration	Number spreading				
10^{-3} *M*	0	0	0	0	0
10^{-4} *M*	0	0	0	0	0
10^{-5} *M*	0	1	5	6	9
10^{-6} *M*	1	2	2	6	8
10^{-7} *M*	0	0	0	0	0
10^{-8} *M*	0	0	1**	0	0
0 *M*	0	0	0	0	0

* Ten isolated gastrozooids were exposed to each concentration.
** Spread only slightly.

A delayed spreading phenomenon was often observed at high concentrations of GSH. For example, 9 of 10 gastrozooids, which initially failed to respond to 10^{-3} *M* GSH, responded 7 hours after the start of the experiment. Occasionally a few gastrozooids took as long as 24 hours to respond. Perhaps these delayed responses occurred when the "excess" GSH had oxidized, giving the optimum concentration.

5. *A comparison of the effects of cysteine and of reduced glutathione*

The effects of another biological reducing agent, cysteine, were tested. The results shown in Table II clearly demonstrate that cysteine failed to induce a feeding response at concentrations at which GSH was active.

6. *Differences in the feeding response of animals in different physiological states*

In one series of experiments the effects of GSH were studied on gastrozooids from two *Physalia* in different physiological states. The first (*ca.* 16-cm. float) was maintained without food in the laboratory for one week, while the second (*ca.* 24-cm. float) was kept under the same conditions for one day. The results in Table III indicate that the one-day captive responded rapidly to 10^{-4} *M* GSH, while the

TABLE II

*A comparison of the effects of cysteine and of reduced glutathione on the spreading of gastrozooids**

Concentration	Number spreading at 90 minutes Cysteine	Reduced glutathione
10^{-4} *M*	1**	0
10^{-5} *M*	0	8
10^{-6} *M*	0	4
10^{-7} *M*	0	2

* Ten isolated gastrozooids were exposed to each concentration.
** Spread only slightly.

7-day captive did not. Also, the response was quicker in the one-day captive animal at all concentrations of GSH. These differences may have been due to the nutritional state or over-all well-being of the one-day captive animal as compared to the 7-day captive animal.

Further studies of more than a dozen *Physalia* revealed striking differences in the responsiveness of the gastrozooids to GSH. Sometimes newly-captured *Physalia* exuded a copious mucus secretion for several days. Gastrozooids from these mucus-covered animals were usually unresponsive to GSH. By contrast, in captives which produced little mucus the gastrozooids always responded. In one case an animal was kept in the laboratory for two weeks and its gastrozooids responded throughout this period.

TABLE III

*A comparison of the effect of different concentrations of reduced glutathione after various time intervals on the gastrozooids of a 7-day captive and 1-day captive Physalia**

Time (min.)	7-day (16-cm. float)		one-day (24-cm. float)	
	10	35	10	35
Concentration	Per cent spreading			
10^{-4} *M*	0	0	66	83
10^{-5} *M*	10	50	33	83
10^{-6} *M*	20	50	50	50–66

* Ten isolated gastrozooids of the 7-day captive and 6 of the one-day captive were used at each concentration.

DISCUSSION

1. *Feeding response*

In *Physalia,* the feeding response of the gastrozooid involves mouth-opening, spreading, and food ingestion. The food is drawn to the gastrozooids by the dactylozooids. Each gastrozooid then writhes and stretches until its mouth comes in contact with a solid object, whether it be the fish that the colony has captured, or the wall of the container in which the gastrozooids were placed. In *Hydra* the tentacles writhe and sweep inward toward the mouth. The mouth then opens and ingests the prey *whenever the prey is brought in direct contact with it* (Ewer, 1947). Thus in contrast to *Physalia* where the whole gastrozooid writhes, in *Hydra* only the tentacles writhe. Moreover, in *Hydra,* the body tube and mouth do not spread when exposed to GSH, but do so only when the food, or some other solid object, is brought to the mouth by the writhing and contracting tentacles. For example, when 10 *Hydra* were placed in each of a series of Petri dishes having 25 ml. of GSH of 10^{-3} to 10^{-7} molarity, none of the *Hydra* exhibited the spreading phenomenon, although their mouths opened. This failure to spread was not due to the tentacles blocking the mouth, because removal of the tentacles did not increase spreading. However, when 10 *Hydra* were crowded in a 0.5-ml. volume of 10^{-4}

M GSH so that their mouths were forced to contact the wall of the container or the surface of the water, 6 animals spread within 3 minutes.

It must be stressed that mere mouth opening or writhing does not indicate a feeding response in *Physalia* or in *Hydra*. The only true index of a feeding response is an actual attempt to feed—that is, to spread the mouth over or around the prey. Many deleterious chemicals cause writhing or mouth opening. For example, in *Physalia,* high concentrations of alloxan (which, besides inhibiting the true feeding response, subsequently killed the gastrozooids) caused occasional "mouth opening" but no characteristic feeding response. This "gaping" has also been observed in *Hydra* by using Tween, and other harmful compounds (Lenhoff, unpublished observations).

2. *The action of reduced glutathione*

The data leave little doubt that GSH induces a feeding response in *Physalia* just as it does in *Hydra*. We cannot be certain that GSH is the only biological compound that will induce the response in *Physalia* but the clear response of the gastrozooids to low concentrations of GSH, coupled with Loomis' finding (1955) that no other commonly occurring compound of many tested worked on *Hydra,* make this likely. Since a gastrozooid of *Physalia* is four to six times as long as a *Hydra,* and thicker, it should be practical to explore the detailed mechanism of the feeding response with greater ease than in the case of *Hydra*. For example, it should be possible to locate the receptor cells sensitive to GSH and to explain the mechanics of the transformation of the cylindrical gastrozooid into a disc.

3. *Phylogenetic considerations*

The Siphonophora are commonly regarded as the most specialized order of the Hydrozoa in that they attain the highest degree of polymorphism and present the greatest number of medusoid and polypoid types. While there is some disagreement regarding the phylogenetic relations within the group, there appears to be no question but that they have clear hydrozoan characters (Hyman, 1940; Totten, 1954). Furthermore it is generally accepted that save for the Chondrophora (*e.g., Velella*) which are now thought to have close affinities with tubularian hydroids (Totten, 1954; Rees, 1957), the Siphonophora proper, including *Physalia,* early diverged from the cnidarian stem and evolved in directions quite different from other hydrozoans (Hyman, 1940). On the other hand, *Hydra* itself is commonly considered a highly specialized gymnoblastic hydroid, in all likelihood a fresh-water-adapted tubularian (Hyman, 1940). Thus among the Hydrozoa it would be hard to find two forms which diverged earlier from one another during evolution—two forms which are very specialized and not generalized members of their class. Recognizing this, the demonstration of a GSH-induced feeding response in both forms assumes special interest. It suggests that either (1) this GSH-induced response is primitive (Loomis, 1955) and has persisted through the course of geological time since these animals diverged because they both retain a primitive carnivorous feeding habit, or (2) that this is simply convergence. The former suggestion is not only more attractive but is more likely, and implies that GSH-stimulation of feeding is a very ancient coordinating system and will be found among many of the

Hydrozoa and possibly among Scyphomedusae and Anthozoa as well. This conclusion finds support in recent observations that *Campanularia flexuosa,* a calyptoblastic hydroid unrelated to either *Hydra* or *Physalia,* gives a feeding response to GSH. It must be emphasized, however, that the presence of a GSH response in these three hydrozoans does not rule out the possibility that other small molecules in the fluids released from captured prey may function in the feeding response of other cnidarians. The essential feature of the primitive feeding mechanism is that the prey must release fluid when pierced (see below).

These experiments support the opinion offered earlier (Schneiderman and Gilbert, 1958) that the evolution of chemical control mechanisms has proceeded by particular groups of animals adapting available and often ubiquitous molecules to special tasks. Under this view the evolution of hormonal coordination involves primarily the evolution of receptor systems sensitive to specific molecules rather than any evolution of hormones as such.

The chemical similarities between the nematocyst-GSH feeding mechanisms of *Physalia* and *Hydra* invite inquiry into the nature of their possible common ancestor. Both of these organisms sting and capture their prey by means of nematocysts. It is of interest in this connection that the nematocyst capsule of *Hydra* (Lenhoff *et al.,* 1957) and *Physalia* (Lane and Dodge, 1958; Lenhoff and Kline, 1958) are composed of similar kinds of unusual hydroxyproline-rich, collagen-like proteins. After the prey is penetrated, the GSH in the fluids flowing from the wound stimulates the feeding response.

Since *Hydra* and *Physalia* both have a nearly identical chemical control of their feeding behavior, and since their nematocysts are of a unique chemical composition, it seems probable that both animals evolved from a common hydrozoan stem-form which also possessed these chemical characteristics. It is noteworthy that the nematocyst-GSH mechanism can only be used to capture prey which has sufficient body fluids to release enough GSH on being stung to elicit the feeding response (*i.e.,* organisms which have either a pseudocoelom, or vascular system) (Loomis, 1955). Therefore, either (1) the primitive nematocyst-bearing hydrozoan stem-form ate prey which was more highly evolved than itself, or alternatively, (2) the primitive nematocyst-bearing hydrozoan stem-form fed on some presently unknown lower form, perhaps a large protozoan, with a great deal of body fluid. We favor the first alternative and prefer to believe that the hydrozoan stem-form fed on animals with "vascular" fluids (*i.e.,* nematodes, and members of higher phyla).

These facts permit us to speculate about the feeding habits of the ancestral cnidarians, *i.e.,* the forms from which the Hydrozoa and the other classes of Cnidaria evolved. Ordinarily one hesitates to base phylogenetic schemes on feeding habits which in most phyla are notoriously labile. However, the universal use of nematocysts to capture prey by all contemporary cnidarians supports the view that the feeding habits of members of this phylum are far more stable than those of other animals. The ancestral cnidarians most probably fed on animals which were of a lower grade of organization than themselves, and hence could not have employed the nematocyst-GSH mechanism. Most likely they were filter feeders like many present-day Anthozoa. Under this view, nematocyst-bearing cnidarians with a GSH-mechanism evolved from filter-feeding ancestors at the same time as did higher forms. Thus cnidarians with nematocysts (a diagnostic feature of the phylum) may be removed from their position as the ancestors of higher metazoans. Whether they are members of a regressive line of evolution which have degenerated because of a sessile habit (Hadzi, 1953), or are an offshoot of a progressive line of evolution, remains to be proven. Since all these events took place in the Pre-Cambrian Era, none of these suggestions can be ruled out at present and doubtless others will arise.

Our special thanks go to the Supply Department of the Marine Biological Laboratory at Woods Hole who went to great efforts to capture the *Physalia* used in this study; to Dr. Sears Crowell for his helpful comments on the typescript of this paper and for his help in conducting experiments with *Campanularia;* and to Mr. Ernest Bay for his most valuable contribution in photographing our observations.

SUMMARY

1. *Physalia* gastrozooids, both isolated and *in situ,* exhibit a feeding response when exposed to low concentrations of reduced glutathione ($10^{-5} - 10^{-6}\ M$).

2. Cysteine did not elicit the response.

3. The feeding response of the gastrozooid consisted of opening of its mouth and the subsequent spreading of its lips over a large area in an attempt to envelop the prey. This response resembles a similar GSH-induced feeding response found in *Hydra.*

4. The sensitivity to GSH depended upon the physiological state of the *Physalia.*

5. The significance of this primitive chemical coordinating mechanism in relation to the evolution of the Cnidaria and of the Metazoa as a whole is considered.

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OBSERVATIONS ON THE GROWTH OF DUNALIELLA EUCHLORA IN CULTURE[1]

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Under natural conditions, an intimate, but poorly understood relationship exists between bacteria and phytoplankton. Some investigators have claimed that phytoplankton produce a substance which is inhibitory to the growth of bacteria (Steemann Nielsen, 1955a, 1955b; Steemann Nielsen and Jensen, 1957). Contrariwise, the results of Waksman *et al.* (1937) suggest a harmonious relationship between algae and bacteria.

In experimental work, the use of bacteria-free cultures is customary. It has not always been possible to obtain bacteria-free cultures, and the assumption has been that the growth of the alga would have not been altered if axenic cultures had been used (*cf.* Goldberg *et al.,* 1951; Kain and Fogg, 1958). This assumption seems correct when using media with no organic additions (McLachlan, unpublished). However, little is known of the effects of bacteria on the growth of algae in cultures containing organic supplements.

In the present investigation, growth of the green flagellate *Dunaliella euchlora* WHOI-1 in pure culture and contaminated cultures containing organic enrichments was studied. Growth was estimated by cell numbers and chlorophyll *a* synthesis. The production of algal and bacterial inhibitors was also investigated.

MATERIALS AND METHODS

A pure culture of *Dunaliella euchlora* Lerche strain WHOI-1 (McLachlan, 1959) was used in this study. The alga was grown in a modification of the ASP medium of Provasoli *et al.* (1957); the composition of this medium is presented in Table I. In some of the experiments, the ASP medium was enriched with organic material by the addition of beef extract (0.3 g./l.) and bactopeptone (0.5 g./l.). At 16% nitrogen, this corresponds to an addition of about 9.1 mM of organic nitrogen, or nine times as much as is available in the basic medium. The cultures were incubated at 16° C. under 3,000 meter-candles of illumination provided by 40-watt fluorescent lights. Growth of the alga was determined by cell counts made in duplicate with a total of eight replicate counts using a Levy hemocytometer, and is expressed as $\log_2 N_t/N_0$ where N_0 is the concentration of cells at inoculation and N_t the concentration at time t.

Chlorophyll *a* was measured spectrophotometrically in acetone extractions according to the Richards with Thompson method (1952) as modified for use with the millipore membrane filter procedure of Creitz and Richards (1955). The con-

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[2] Public Health Service Research Fellow of the National Institutes of Health.

TABLE I

Composition of the modified ASP medium

NaCl	410 mM	$FeCl_3 \cdot 6H_2O$	1.5 μM
$MgSO_4 \cdot 7H_2O$	24 mM	H_3BO_3	185.0 μM
$MgCl_2 \cdot 6H_2O$	22 mM	$MnCl_2 \cdot 4H_2O$	7.0 μM
$CaCl_2 \cdot 2H_2O$	10 mM	$ZnCl_2$	0.8 μM
KNO_3	1 mM	$CoCl_2 \cdot 6H_2O$	0.02 μM
K_2HPO_4	100 μM	$CuCl_2 \cdot 2H_2O$	0.0002 μM
$Na_2SiO_3 \cdot 9H_2O$	100 μM	Na_2EDTA	30.0 μM

centration of chlorophyll *a* was determined using the nomographs of Duxbury and Yentsch (1956), and the organic nitrogen content of the cells was estimated by the procedure of Yentsch and Vaccaro (1958).

RESULTS

1. *Growth in contaminated beef extract-peptone cultures.* To determine the effect of bacterial contamination on the growth of *Dunaliella euchlora,* the alga was inoculated into autoclaved and unautoclaved ASP medium, and autoclaved and

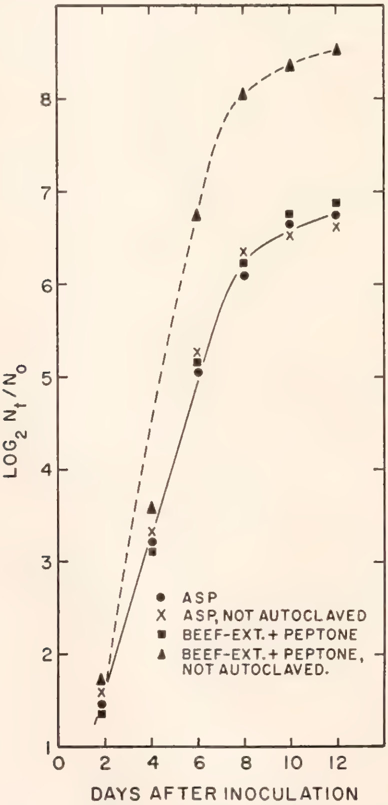

FIGURE 1. Growth of the alga in contaminated and non-contaminated ASP medium and contaminated and non-contaminated ASP medium containing beef extract and peptone.

unautoclaved ASP cultures containing beef extract and peptone. In the unautoclaved cultures with organic enrichments, approximately 2.5 times as many cells were obtained as in the other cultures (Fig. 1). The growth rate and the final number of cells in the other treatments were approximately the same. From these results it can be seen that the addition of beef extract and peptone *per se* did not increase the growth of the alga, but bacterial breakdown of these materials was of considerable benefit.

The unautoclaved cultures with organic additions became very dense, and after twelve days of growth contained approximately three times as much chlorophyll *a* per cell as the autoclaved enriched culture (Table II). Using the method of Yentsch and Vaccaro (1958), it was found that in the enriched contaminated cultures about 10 mM per liter of organic nitrogen was incorporated into the algal cells, or approximately 0.91×10^{-6} μM of nitrogen per cell. In contrast, the non-contaminated enriched culture contained about 0.9 mM per liter of organic nitrogen as algal cellular material, or approximately 0.23×10^{-6} μM of nitrogen per cell. The chlorophyll content of the two cultures containing only inorganic additions was not determined, but other estimates have indicated that at the end of exponential growth, all of the added nitrate-nitrogen is organically incorporated. This suggests

TABLE II

The size of the population of Dunaliella euchlora, *and the chlorophyll* a *content after growth in media treated as discussed in the text*

Experiment	Treatment	Days of growth	No. cells/ml. $\times 10^4$	Mg Chl^a/cell $\times 10^{-9}$
Beef extract-peptone no. 1	Enriched and contaminated	12	1,089	2.24
	Enriched, not contaminated	12	397	0.74
	ASP-autoclaved	12	384	—
	ASP-not autoclaved	12	370	—
Nitrate-nitrogen	1.0 mM	14	444	0.41
	2.0 mM	14	868	0.40
	5.0 mM	14	839	0.44
	8.0 mM	14	716	0.39
	10.0 mM	14	617	0.51
Beef extract-peptone no. 2	−3	15	320	0.81
	0	15	634	0.66
	+2	15	924	0.47
	+4	15	1,631	0.60
	+7	15	1,198	1.11
	+11	15	792	0.68
	Control	15	497	0.79
Dunaliella filtrate	ASP	11	504	0.44
	Autoclaved filtrate	11	527	0.93
	Unautoclaved filtrate	11	491	1.27
Bacteria filtrate	ASP	12	477	0.43
	Filtrate	12	958	0.80

that in the non-contaminated beef extract peptone culture all of the incorporated organic nitrogen had been obtained from the added nitrate-nitrogen, and not from the organic material.

2. *Growth in various concentrations of nitrate-nitrogen.* To determine if the number of cells and the amount of chlorophyll *a* obtained in the contaminated beef extract-peptone cultures could be obtained by the addition of inorganic nitrogen, sodium nitrate was added at the following concentrations: 1.0 (ASP level), 2.0, 5.0, 8.0 and 10.0 mM. The ASP concentration of potassium was maintained by the addition of potassium chloride, and the phosphorus concentration was maintained at 100 μM in all cultures. The two highest concentrations of nitrogen slightly inhibited the growth of the alga (Fig. 2), but the total number of cells in all cultures exceeded that of the 1.0 mM culture after fourteen days of growth. The maximum density of cells developed in the 2.0 and 5.0 mM cultures had a final cell count of almost twice that of the 1.0 mM culture (Table II), although the initial growth rates of these three cultures were approximately the same.

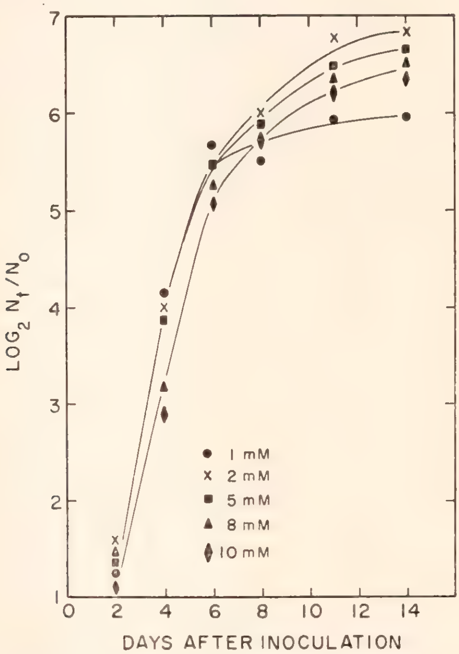

FIGURE 2. Growth of the alga in various concentrations of nitrate-nitrogen.

Pigment analysis showed that the chlorophyll *a* content of the cells in all cultures was about the same (Table II). The total number of cells obtained in the cultures containing 2.0 and 5.0 mM of nitrate-nitrogen approached that obtained in the contaminated beef extract-peptone culture of the previous experiment, but the amount of chlorophyll *a* was considerably less. Since the maximum amount of chlorophyll synthesized in these cultures was considerably less than was found in the contaminated culture containing beef extract and peptone, it may be concluded that available nitrogen alone is not the limiting factor. Perhaps release of other nutrients in beef extract and peptone by bacterial activity or direct utilization of organic breakdown products by the alga also stimulated chlorophyll synthesis.

3. *Growth in beef extract-peptone cultures with periodic addition of bacteria.* To determine the effects of organic breakdown products on the growth of the alga at different phases of growth, a mixed culture of marine bacteria was obtained from the surface waters of Woods Hole Harbor, and uniform inocula were added periodically to cultures of *Dunaliella* containing beef extract and peptone. The original bacterial inoculum was collected using a sterile container so only bacteria present in the surface water were introduced into culture. The bacteria were grown in the ASP medium with the addition of beef extract and peptone under the same conditions as the algal cultures. Before the bacterial cultures were used in the experimental work, they were carried through a number of transfers in order to obtain a uniform population.

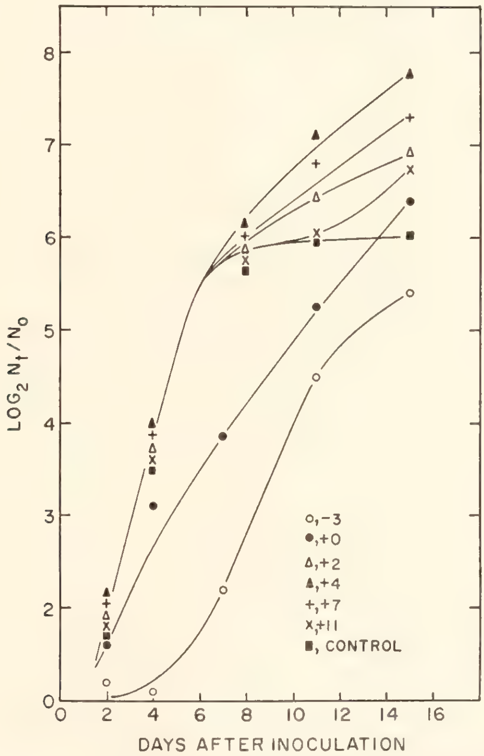

FIGURE 3. Growth of the alga in the ASP medium containing beef extract and peptone to which bacteria were added periodically.

Beef extract and peptone were added to the ASP medium and inoculated periodically with 1 ml. of the bacterial culture. In all cases the bacteria inocula were from three-day-old cultures when introduced into the experimental flasks. Inoculation

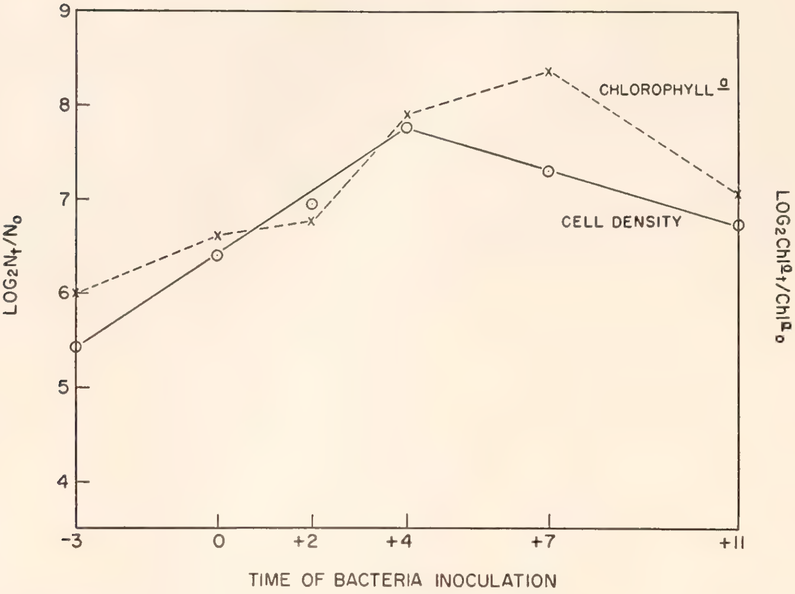

FIGURE 4. A comparison of algal cell density and chlorophyll *a* in cultures to which bacteria were added periodically.

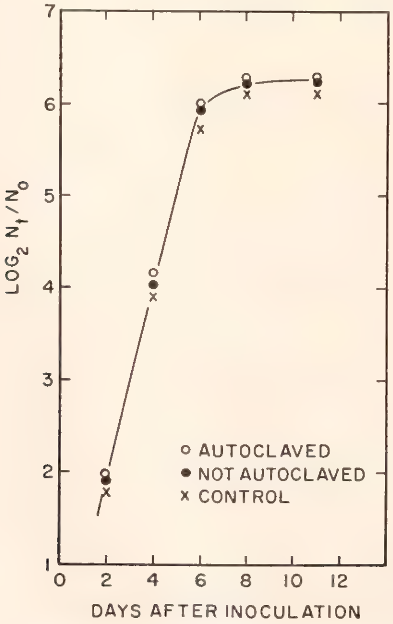

FIGURE 5. Growth of the alga in autoclaved and non-autoclaved algal filtrate.

took place at the following times: three days before inoculation with the alga, at the same time as the alga, two days, four days, seven days, and eleven days after inoculation with the alga.

Bacteria introduced three days before and at the time of inoculation with *Dunaliella* inhibited the growth of the alga (Fig. 3). In all cultures, except the one inoculated with bacteria three days before the alga, growth after fifteen days exceeded that of the control. In Figure 4 cell density and chlorophyll *a* after fifteen days of growth are presented. The greatest number of cells occurred in the culture which was inoculated with bacteria four days after the alga, and those cultures which were inoculated with bacteria prior to this time showed less growth. Less growth was also obtained in the cultures which had been inoculated with bacteria seven and eleven days after inoculation with the alga. However, the cells in the culture inoculated with bacteria seven days after the alga contained more chlorophyll *a* than the other cultures after fifteen days of growth (Table II). These results show that growth of the alga was directly related to the time at which the bacteria were added. Under the conditions of this experiment, chlorophyll *a* synthesis preceded cell division (Fig. 4). The highest concentration of chlorophyll per cell, however, was not as great as that obtained in the previous beef extract-peptone experiment.

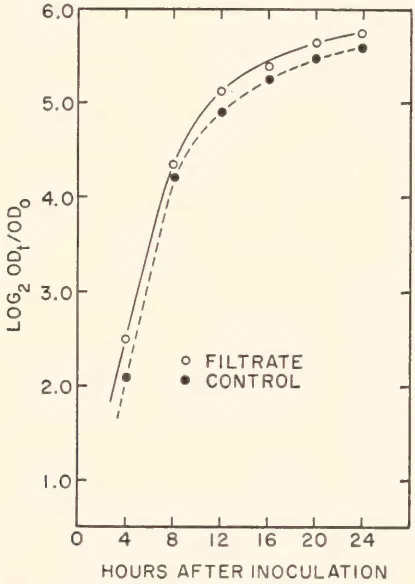

FIGURE 6. Growth of Woods Hole Harbor bacteria in the algal filtrate containing beef extract and peptone.

4. *Growth of the alga in the algal filtrate.* To determine if auto-inhibitors are produced by *Dunaliella euchlora,* growth of the alga in the ASP medium was compared with growth in the filtrate of a *Dunaliella* culture which had reached maximum density. Part of the filtrate was autoclaved and the other part was not

autoclaved as there has been a suggestion of heat-labile inhibitors (Lefèvre *et al.*, 1952). Nitrogen, phosphorus, and iron were added to all cultures at ASP concentrations. Growth in all cultures was identical as shown by the data in Figure 5. The chlorophyll *a* content per cell was greater in the filtrate cultures than in the control whether or not the filtrate had been autoclaved (Table II). This indicates that the filtrate contained something which enhanced chlorophyll synthesis, but did not promote cell division.

5. *Growth of the bacteria in the algal filtrate.* To determine if *Dunaliella* filtrate would inhibit the growth of bacteria, a filtrate was obtained from a five-day culture of the alga. Beef extract and peptone were added to the filtrate and to the control ASP medium, and inoculated with the previously isolated Woods Hole Harbor bacteria. Growth of the bacteria was measured by optical density at 750 mμ using a Beckman model DU spectrophotometer, and is expressed as $\log_2 OD_t/OD_o$. The growth of the bacteria was approximately the same in the filtrate as in the enriched ASP medium with no suggestion of a bacteriostatic substance produced by the alga (Fig. 6).

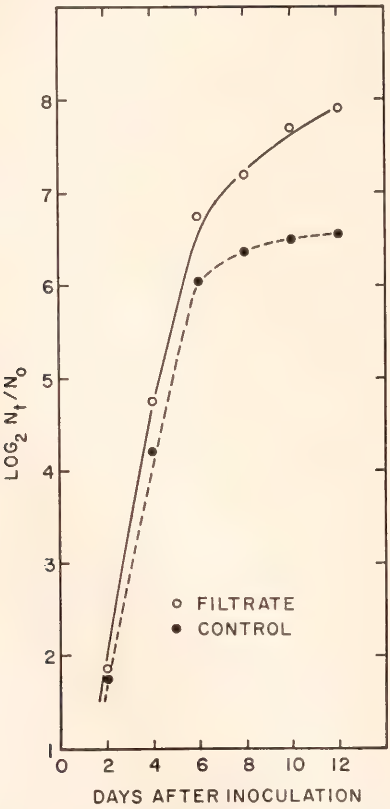

FIGURE 7. Growth of the alga in the Woods Hole Harbor bacteria filtrate.

6. *Growth of the alga in the bacterial filtrate.* To determine the effects of the bacterial filtrate on the growth of *Dunaliella,* a culture of the Woods Hole Harbor bacteria was grown in the ASP medium containing beef extract and peptone. After three days of growth, the culture was passed through a HA millipore filter resulting in a clear filtrate which was autoclaved and inoculated with the alga. There was a considerable increase in the maximum cell density in the filtrate culture as compared with the control (Fig. 7). The inhibition noted in the previous beef extract-peptone experiment where the bacteria had been added to the culture three days before the alga did not occur in this experiment. This suggests that the inhibitory substance is volatile or heat-labile. Pigment analysis also showed the cells in the filtrate culture contained considerably more chlorophyll *a* than those in the control culture (Table II).

DISCUSSION

The addition of beef extract and peptone to bacteria-free cultures of *Dunaliella euchlora* did not stimulate the growth of the alga. In the presence of bacteria, however, a considerable increase in the algal population, as determined by cell counts and chlorophyll analysis, was observed. This increase presumably resulted from the bacterial hydrolysis of the added organic material. The organic additions were not entirely inert to algal metabolism as the amount of chlorophyll *a* per cell in the uncontaminated cultures always exceeded that obtained with only the addition of inorganic salts.

The large amount of synthesis of chlorophyll *a* suggested that the alga was able to utilize nitrogenous breakdown products of the peptone and beef extract. A large fraction of the utilizable nitrogen from the beef extract and peptone was probably present as ammonia, nitrite, or simple organic compounds since the conversion of these to nitrate-nitrogen by marine bacteria is a slow process (Harvey, 1955; Sverdrup *et al.,* 1942). Harvey (1940) and Gibor (1956) observed that several species of *Dunaliella* were able to utilize some amino acids as nitrogen sources. There are also numerous reports of other autotrophic algae capable of utilizing organic nitrogen compounds (Fogg, 1953; Ryther, 1954). In addition, Huzisige *et al.* (1957) found that *Euglena* synthesized chlorophyll more rapidly when provided with casein hydrolysate than when provided with inorganic nitrogen compounds.

Growth of the alga in cultures containing organic additions was inhibited if bacteria were introduced before the alga began to grow. However, no inhibition of the alga was observed in the bacterial filtrate which had been autoclaved. This suggests a volatile or heat-labile inhibitor. Inhibition could have resulted from ammonia which is known to be toxic at low concentrations (Fogg, 1953; Myers, 1951). Any ammonia present in the bacterial filtrate would have been lost during autoclaving, and the inhibition noted in the second beef extract-peptone experiment may have been due to toxic concentrations of ammonia.

In the cultures which developed a heavy suspension of cells and chlorophyll, light and probably carbon dioxide may have been limiting, but organic carbon could have been utilized by the alga. Organic carbon could have been incorporated by the alga simultaneously with the utilization of organic nitrogen (Fogg, 1953; Krauss, 1958), or as separate compounds. If the alga is capable of heterotrophic growth, light would not necessarily be a limiting factor. It seems probable that the very dense cultures obtained in this study may have been due in part to heterotrophic growth.

It was not possible in this study to demonstrate that *Dunaliella* inhibited the growth of bacteria, nor was there any indication of auto-inhibitors produced by the alga. The production of antibiotics by algae may not be a general phenomenon.

The authors wish to express their grateful appreciation to Dr. Bostwick H. Ketchum for his many helpful suggestions in preparing the manuscript.

SUMMARY

1. Bacteria added to cultures of *Dunaliella euchlora* in the presence of nitrogenous organic matter stimulated the growth of the alga, and enhanced the synthesis of chlorophyll *a*.

2. It was not possible to obtain comparable concentrations of chlorophyll *a* by the addition of nitrate-nitrogen, although a comparable number of cells could be obtained.

3. If the bacteria obtained a "head-start" in the enriched cultures, growth of the alga was inhibited. This inhibition could be overcome by autoclaving the filtrate from the bacteria cultures.

4. Growth of the bacteria was not inhibited in the algal filtrate, nor was the growth of the alga inhibited in the algal filtrate.

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MARINE BIOLOGICAL LABORATORY

INHIBITION OF FERTILIZIN AGGLUTINATION OF SPERM BY THE DERMAL SECRETION FROM ARBACIA[1]

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Upon appropriate stimulation, *Arbacia* release a yellowish fluid from the integument. This fluid strongly inhibits fertilization (Oshima, 1921; Pequegnat, 1948) and is probably the material responsible for the inhibitory action ascribed to *Arbacia* blood by Lillie (1914). Current interest arose from the observation that this "dermal secretion" not only inhibits fertilization but inhibits fertilizin agglutination of sperm as well. Fertilizin is the specific sperm isoagglutinin obtained from eggs of the species. It is initially present as a jelly surrounding the sea urchin egg, but on standing in sea water this jelly dissolves charging the water with the agglutinin. The dual effect of the dermal secretion suggests a causal relation between inhibition of fertilizin agglutination of sperm and of fertilization. Demonstration of such a relation would support the view that fertilizin is essential for fertilization. The experiments described here and reported briefly elsewhere (Metz, 1958) mainly concern the action of the dermal secretion on sperm and the fertilizin agglutination of sperm. The results are consistent with the view that inhibition of fertilization by the dermal secretion is related to an inhibitory action on fertilizin.

MATERIALS AND METHODS

Arbacia punctulata was used in all experiments except the specificity tests outlined in the text. Most of this material was collected in the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida, although a few collections were made from a very large population at Panama City, Florida. A few experiments were performed at the Marine Biological Laboratory, Woods Hole, Mass., using the local material.

Gametes were obtained by electrical stimulation of the animals. Gametes obtained by the KCl injection method, especially during the winter months, proved unsatisfactory in both fertilization and sperm agglutination tests (Metz, 1957a). In fact, investigation of the cause of this failure revealed that KCl-treatment frequently stimulated release of the dermal secretion (see also Harvey, 1956, page 57) as well as the gametes.

Sperm oxygen consumption was measured using standard Warburg apparatus. Single side arm vessels of 15–20 ml. capacity containing 3 ml. of fluid were run in duplicate at 20° C. The vessels were shaken at a rate of 120 cycles per minute with an amplitude of 3.5 cm.

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Solutions of the dermal secretion were prepared in a manner similar to that described by Pequegnat (1948), and consisted of the following steps: The *Arbacia* were immersed in tap water for 1–3 minutes. They were subsequently rinsed in distilled water and finally in filtered sea water. The animals were then placed in a dry funnel and the yellow fluid which drained off was collected. The pH of such fluid is about 7.5. In experiments where this might be critical the pH was raised to that of sea water (pH 8.0–8.2). To test for sperm agglutination one to two drops of the test solution were mixed with an equal volume of a sperm suspension prepared by diluting semen to 2% with sea water. The mixtures were examined both macro- and microscopically.

RESULTS

A) *Action on sperm*

Pequegnat (1948) noted that the dermal secretion of *Arbacia* stimulated sperm to increased activity. This effect was observed repeatedly in the current investigation. Even preparations of dermal secretion at the initial pH (7.5), and therefore at a pH disadvantage as compared to sea water controls, had the stimulating action. As Pequegnat was aware, this effect is not a transient one. The sperm remain strongly active for a prolonged period of time.

The enhanced motility of the sperm in the presence of the dermal secretion is associated with an increase in oxygen consumption as seen in Figure 1. Again the effect is not a short term one for the inhibitor-treated sperms were still respiring at approximately twice the rate of the controls even at the end of one hour. The increased oxygen uptake is clearly the result of enhanced sperm respiration, not to the oxidation of the pigment in the dermal secretion (see later section). This follows from the fact that the increase in oxygen uptake does not appear until the dermal secretion is tipped into the sperm suspension. Furthermore, in independent experiments dermal secretion collected over nitrogen to prevent premature oxidation failed to consume appreciable amounts of oxygen in Warburg vessels.

Nothing definite is as yet known regarding the chemical nature or properties of the sperm-stimulating agent, except that it is gradually destroyed by heat (Fig. 1).

B) *Action on fertilizin agglutination of sperm*

An inhibitory effect of the dermal secretion on fertilizin agglutination of sperm was first noted by Pequegnat (1948). The present investigation was designed to obtain information concerning the mechanism of action of the material in this effect. Attention was directed especially to determine if the inhibitory agent acted upon the sperm, the fertilizin or both constituents of the agglutinating system.

Possible inhibitory action on the sperm. One possible means whereby the dermal secretion could inhibit agglutination would be by action on the sperm. For example, the agent could destroy the antifertilizin receptor sites on the sperm surface. Such destruction might be expected to be irreversible in which case washing dermal secretion-treated sperm should not restore agglutinability. Such experiments show, however, that treated sperm recover agglutinability upon washing in sea water. A typical experiment from a series of four is given in Table I. From this experiment it is clear that the inhibiting agent in the dermal secretion does not render the sperm irreversibly refractory to the agglutinating action of fertilizin.

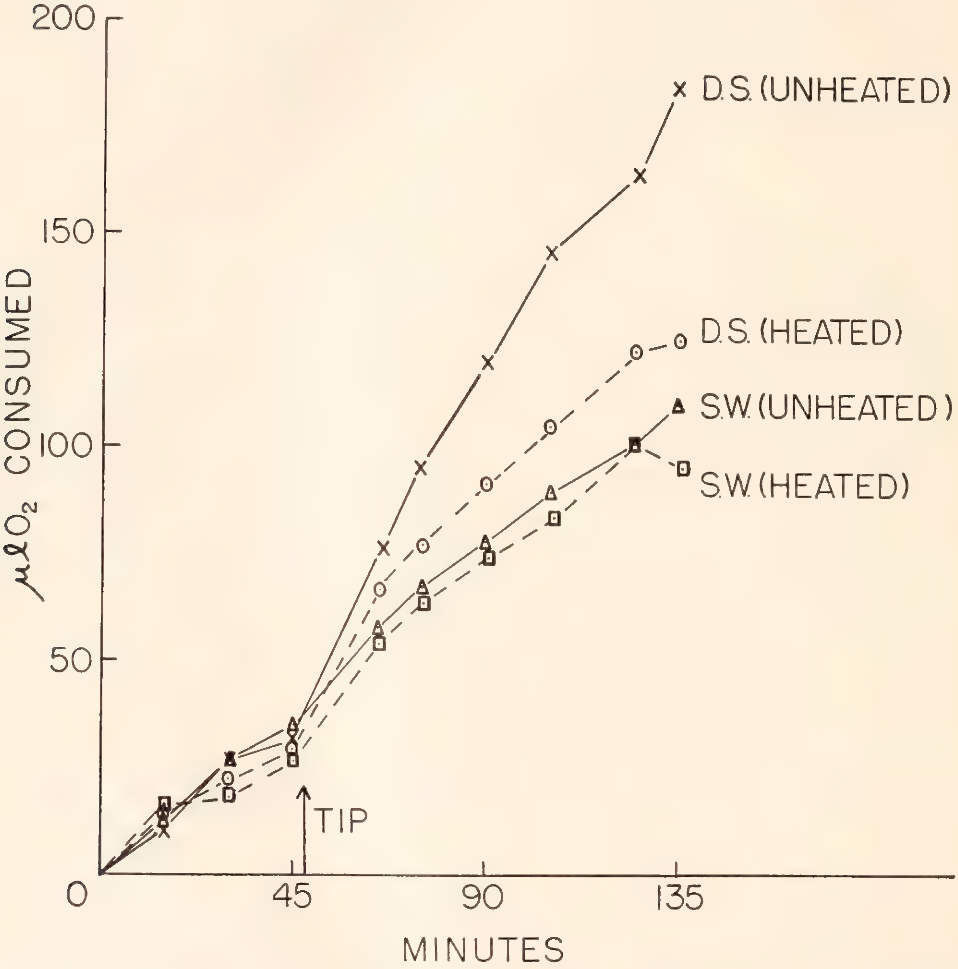

FIGURE 1. Effect of dermal secretion (D.S.) on oxygen uptake of *Arbacia* sperm. Heated aliquots (3 ml.) were immersed in a boiling water bath for 5 minutes. The pH of both heated and unheated dermal secretion solutions was adjusted to 7.9. Vessels contained 1.8 ml. of *Arbacia* semen diluted to 10% with sea water. Center wells contained 0.2 ml. 20% KOH. The 1-ml. samples of dermal secretion (or sea water) in side arms were tipped into vessels at 48 minutes.

Likewise, the inhibitor is not used up or destroyed by sperm in the inhibiting reaction as seen in Table II (one of five experiments). Accordingly, it appears likely that the dermal secretion acts upon the fertilizin, not the sperm, to inhibit agglutination. Experiments supporting this view are described below.

TABLE I

Fertilizin agglutination of Arbacia sperm after washing from dermal secretion

	1	2	3	4
	Sperm + D.S. (Sperm re-suspended in S.W.)	Sperm + D.S. (Sperm re-suspended in original supernatant D.S.)	Sperm + S.W. (Sperm re-suspended in S.W.)	Sperm + S.W. (Sperm re-suspended in original supernatant S.W.)
Agglutination of sperm on addition of fertilizin after one centrifugation	+	−	++++	++++
Agglutination of sperm on addition of fertilizin after second centrifugation	+++	−	++++	++++

Semen concentration in the four 0.75-ml. samples was 8%. After standing 30 minutes the samples were centrifuged twice at 4° C. Sperm in 1 and 3 was re-suspended in sea water; the sperm in 2 and 4 in the original supernatant after each centrifugation. Aliquots of sperm from all re-suspended samples were tested for agglutination with fertilizin.

Inhibitory action on fertilizin. Tests for action of the inhibiting solution on fertilizin are complicated by the fact that fertilizin itself can inhibit sperm agglutination when in the univalent form (Metz, 1942). Indeed, one possible mode of action of the inhibitor would be that it converts fertilizin from the multivalent, agglutinating form to the univalent, non-agglutinating condition. Another pos-

TABLE II

Agglutination inhibiting action of sperm absorbed dermal secretion

	Absorbing mixtures			
	1	2	3	4
0.5 ml. 0.5 ml.	Sperm (33%) + D.S.	S.W. + D.S.	Sperm (33%) + S.W.	S.W. + S.W.
	Agglutination inhibiting assay of supernatants from above mixtures			
Dilution of absorption supernatant	1	2	3	4
1	−	−	++++	++++
$\frac{1}{2}$	−	−		
$\frac{1}{4}$	±	−		
$\frac{1}{8}$	+++	++		
$\frac{1}{16}$	+++	+++		

After standing for 30 minutes the absorbing mixtures were centrifuged, supernatants were removed and diluted serially in S.W. Constant amounts of fertilizin and test sperm suspension were then added to each dilution. The slight difference between No. 1 and No. 2 is not considered significant.

sibility is that the inhibitor destroys the reactive sites of the fertilizin which combine with the sperm surface. This could occur with or without conversion to the univalent form.

Ideally, to test for direct action on fertilizin, the inhibiting agent and fertilizin should be mixed and subsequently separated before testing the fertilizin for activity. Unfortunately, no simple means has yet been devised for separating the two materials. However, results obtained using two other procedures leave no doubt that the agglutination-inhibiting agent does destroy not only the sperm-agglutinating action of fertilizin, but also the ability of the fertilizin to combine with the antifertilizin of the sperm.

In the first procedure advantage was taken of the fact that the agglutination-inhibiting action of the dermal secretion is rapidly destroyed by heating to 100° C. whereas fertilizin is relatively stable to such heating. As seen in Table III (A) when a non-agglutinating, fertilizin-inhibitor mixture was heated sufficiently to destroy the inhibitor but not the fertilizin, the mixture still failed to agglutinate sperm. Restoration of agglutinating action would be expected if the inhibitor in the mixture acted exclusively on the sperm. Accordingly, the experiment is explained by an inactivation of the fertilizin by the inhibitor and a subsequent inactivation of any remaining inhibitor by the heating. Four other experiments yielded similar results.

TABLE III

Effect of heating on fertilizin-dermal secretion mixtures

	Heated mixtures				Unheated mixtures			
	1	2	3	4	5	6	7	8
0.5 ml. 0.5 ml.	D.S. + fertilizin	D.S. + S.W.	S.W. + fertilizin	S.W. + S.W.	D.S. + fertilizin	D.S. + S.W.	S.W. + fertilizin	S.W. + S.W.
A. Agglutination on addition of sperm to mixtures	–	–	++++	–	–	–	++++	–
B. Inhibition tests (agglutination on addition of control fertilizin to samples tested in *A* above)	+++	+++	–	+++	–	–	–	+++

The heated mixtures were held at 100° C. for four minutes. In *A* the mixtures were tested for agglutinating action on sperm. The heating failed to restore agglutinating action to the D.S.-fertilizin mixture (*A1*). In *B* the mixtures were tested for agglutination inhibiting action by adding control fertilizin to the mixtures in *A* following spontaneous reversal of the initial agglutination in *A3* and *A7*. The heated mixture fails to inhibit agglutination (*B1* and *B5*). Both the inhibitor in the dermal secretion and the fertilizin have been destroyed in the heated mixture.

The question now arises whether the agent inactivates the sperm combining sites of fertilizin or converts the fertilizin to the univalent form without combining site destruction. Evidence concerning this was obtained by testing for sperm agglutination inhibiting action of the heated fertilizin-inhibitor mixture. As seen in Table III (B) control fertilizin agglutinates sperm that was pretreated with the heated fertilizin-inhibitor mixture. This agglutination means that the heated mixture contains insufficient fertilizin combining sites to block the sperm surface. However, controls in the experiment show that fertilizin combining sites sufficient to block the sperm surface were initially present in the mixtures. Therefore, it must be concluded that combining site destruction occurred in the fertilizin-inhibitor mixture. Inactivation of agglutinating action by dermal secretion does not result simply from conversion of the fertilizin to the univalent form.

In a second series of experiments fertilizin was tested for its ability to combine with the sperm surface in the presence of unheated dermal secretion. As seen in Table IV sperm was mixed with a non-agglutinating inhibitor-fertilizin mixture in proportions that assured sufficient fertilizin to saturate the sperm surface. The sperm in the mixture was subsequently centrifuged free of the mixture and tested for agglutinability by control fertilizin. As seen in the table the sperm washed from the fertilizin-inhibitor mixture agglutinated upon addition of control fertilizin, but sperm washed from fertilizin alone failed to agglutinate. In the latter case, the fertilizin evidently saturated the sperm surface (as univalent fertilizin) to prevent agglutination by the control fertilizin. Failure of fertilizin to block the sperm surface in the fertilizin-inhibitor mixture is most readily explained by an inactivation of combining sites of fertilizin by the inhibitor prior to addition of sperm.

TABLE IV

Fertilizin agglutination of Arbacia sperm washed from fertilizin-dermal secretion mixtures

	1	2	3	4
Sperm washed from mixture of.....	D.S. + fertilizin	D.S. + S.W.	S.W. + fertilizin	S.W. + S.W.
Agglutination on addition of fertilizin to the washed sperm	+++	++++	−	++++

The four mixtures were prepared in the following proportions: 0.5 ml. dermal secretion, 0.25 ml. fertilizin, 0.25 ml. approximately 47% semen. They were centrifuged at 4° C., and the sperm were re-suspended in 0.5 ml. sea water. These sperm suspensions were then tested for agglutinability with control fertilizin. The sperm suspensions were not agglutinated in sea water alone. Test 3 shows that the original mixtures 1 and 3 contained sufficient fertilizin to block the sperm surface. In the presence of dermal secretion, this amount of fertilizin failed to block the sperm surface (test 1). Controls not listed show that the mixtures contained an excess of dermal secretion.

C) *Action on antifertilizin agglutination of eggs*

In view of its action on solutions of fertilizin the dermal secretion might be expected to affect fertilizin in the gel form. The natural jelly surrounding the eggs of sea urchins is largely, if not entirely, fertilizin (Tyler, 1949). Thus the dermal secretion might be expected to precipitate, dissolve or otherwise alter this jelly layer in some visible way. In any event it should affect the egg agglutination and jelly precipitation reaction that results when antifertilizin from sperm is mixed with eggs.

According to Pequegnat (1948) (p. 79) the dermal secretion "appeared to remove part of the egg's jelly layer, in proportion to concentration or to the duration of exposure." Similar action was sometimes observed in the present investigation. However, the effect was not consistently found. It seems likely that this jelly-dissolving action is related to the pH of the inhibitor solutions. Aside from this possible dissolving action the dermal secretion has no visible effect on the egg jelly. It certainly does not precipitate the egg jelly in the form of a membrane as might be expected in view of its ability to inactivate the combining sites of fertilizin.

In spite of its failure to have a direct precipitatory action on the egg jelly, the dermal secretion was found to inhibit the jelly precipitation and egg agglutination that results when sperm antifertilizin is mixed with eggs. However, the inhibiting action on egg jelly precipitation was found to be reversible. After washing the eggs from the dermal secretion or dermal secretion-antifertilizin mixture into sea water, and subsequently adding antifertilizin they formed satisfactory jelly precipitation membranes. Clearly, then, the dermal secretion inhibits antifertilizin agglutination of eggs. However, the mechanism of this action is apparently more complicated than would have been predicted from the mode of action of the dermal secretion on sperm agglutination. Results from the latter study (above) indicate that the dermal secretion inactivates the combining sites of fertilizin. The inhibition of egg agglutination by antifertilizin, however, appears to result primarily from action of the material on the egg agglutinating agent, antifertilizin, in the sperm extracts. This conclusion is suggested by 1) the ready reversibility of the inhibition upon washing eggs, 2) the fact that a precipitate forms upon mixing antifertilizin and the dermal secretion and finally, 3) the fact that the egg agglutination

TABLE V

Effect of heated and unheated dermal secretion on A, fertilizin agglutination of sperm and B, antifertilizin precipitation of egg jellies

	A				B			
	D.S. + fertilizin + sperm	D.S. + S.W. + sperm	S.W. + fertilizin + sperm	S.W. + S.W. + sperm	D.S. + anti-fertilizin + eggs	D.S. + S.W. + eggs	S.W. + anti-fertilizin + eggs	S.W. + S.W. + eggs
Unheated D.S.	–	–	++++	–	–	–	++++	–
Heated D.S.	++++	–	++++	–	–	–	++++	–

The heated dermal secretion was treated for 5 minutes at 100° C. Two other experiments gave similar results. In one of these the dermal secretion was heated for 25 minutes.

inhibitor is heat-stable, whereas the fertilizin agglutination inhibitor is heat-labile. These last relations are demonstrated in the experiment summarized in Table V. From these experiments it would appear that there are two inhibitors in the dermal secretion. One of these inhibits the sperm agglutinating action of fertilizin by inactivating the combining sites of the agglutinin. The second agent inhibits the egg jelly precipitating and agglutinating action of sperm antifertilizin by inactivating the antifertilizin.

Although these experiments adequately explain failure of antifertilizin to agglutinate eggs in the presence of dermal secretion, they still do not directly answer the question whether the heat-labile agent can combine with the reactive sites of fertilizin when the latter is in the gel form. To test for such combination, eggs were treated with an excess of dermal secretion for periods up to 55 minutes. The eggs were subsequently washed in sea water and extracted for fertilizin in normal or acid sea water. In each of three such experiments the dermal secretion-treated eggs yielded sperm agglutinating fertilizin solutions. Indeed the sperm agglutinin titers of fertilizin solutions prepared by acid extraction of dermal secretion-treated and control eggs were not significantly different. Evidently, then, the reactive sites of fertilizin in the gel form are not accessible to the heat-labile inhibitor of the dermal secretion.

D) *Some physical and chemical properties of the dermal secretion*

No systematic study of the physical and chemical properties of the dermal secretion has yet been made. Nevertheless, some information has been obtained concerning the secretion and the sperm agglutination inhibitor contained in it. This information is recorded here.

Color changes. The freshly prepared dermal secretion is light yellow-green in color. Upon standing in air the solution gradually darkens to a deep brown or black color. This color change is evidently a rather direct oxidation by atmospheric oxygen, for the color change fails to occur in an atmosphere of nitrogen. Furthermore, it appears unlikely that the oxidation is mediated by enzymes because both heated and unheated preparations undergo the color changes. Finally, the oxidation is not reversed by reducing agents such as hydrosulfite. The colored material is a component of a large molecule since it precipitates with $(NH_4)_2SO_4$ and fails to diffuse through cellophane. The sperm agglutination inhibitor is associated with the pigmented material to the extent that the inhibitor also precipitates with $(NH_4)_2SO_4$ and fails to diffuse.

Antigenic composition. No serious serological study has been made of the dermal secretion. Nevertheless, the dermal secretion lowered the sperm agglutinating titer of anti-*Arbacia* sperm serum. In agar diffusion precipitin tests (Ochterlony tests) the dermal secretion produced three precipitin bands both with antisera prepared against *Arbacia* sperm and with antisera against jellyless *Arbacia* eggs. One precipitin band formed when the dermal secretion was diffused against antiserum prepared against *Arbacia* fertilizin. No precipitates formed with control (pre-injection) serum. The antisera used in these experiments were prepared with considerable care. The sperm and jellyless eggs used for injection were washed to remove contaminating material. Likewise, the fertilizin used as immunizing antigen was obtained by careful acid extraction of washed eggs (see Tyler, 1949).

In view of this, it appears likely that the immunizing antigens were largely free of contaminating material including dermal secretion. Evidently, then, the dermal secretion contains antigenic groups in common with sperm, eggs and fertilizin. The antigenic relationships here have not yet been investigated, but it is evident that the dermal secretion contains at least three separate and distinct materials.

DISCUSSION

The observations of Oshima (1921) and Pequegnat (1948) combined with the present investigation show that the dermal secretion of *Arbacia* has a variety of effects on sea urchin gametes and their interaction. Action on sperm includes enhancement of motility and respiration. These are not pH effects but appear to depend upon an agent or agents in the dermal secretion. The two effects may be due to separate agents although it seems more likely that increase in motility and respiration are related and result from action of a single agent. Little information concerning the mechanism of action of the agent has been obtained. However, it is unlikely that the motility and respiration enhancing effects are due to a metal-binding action of the dermal secretion. Metal-binding agents do increase the motility of sea urchin sperm (Tyler and Atkinson, 1950), but they differ from the dermal secretion in that they fail to increase the rate of oxygen uptake (Tyler and Rothschild, 1951; Tyler, 1953). Metal-binding agents and the dermal secretion of *Arbacia* also differ in their action on *Asterias* sperm. The former agents have a spectacular stimulatory action on *Asterias* sperm motility (Metz and Birky, 1955) whereas the dermal secretion of *Arbacia* was observed to have no effect on *Asterias* sperm motility. Evidently, the dermal secretion does not contain appreciable amounts of substances which bind metals. Finally, contamination with the dermal secretion is a hazard to be avoided in studies of stimulating action of egg water and other extracts on sperm motility and respiration.

The dermal secretion also inhibits fertilizin agglutination of sperm, antifertilizin precipitation of egg jelly and fertilization. From the sperm centrifugation experiments it is evident that the dermal secretion does not inhibit agglutination by combining with and blocking the sperm surface in irreversible fashion. In this respect the agent differs from fertilizin. As is well known, sea urchin sperm washed from an excess of fertilizin after reversal of agglutination fails to reagglutinate upon a second addition of fertilizin. Indeed, sperm remove the fertilizin from solution (*e.g.,* Monroy *et al.,* 1954). Conceivably, the agent could inhibit agglutination enzymatically by digesting the antifertilizin from the sperm surface with sufficient rapidity to prevent agglutination. If the antifertilizin were arranged in layers at the sperm surface, sufficient of this material might remain after addition and subsequent removal of the inhibitor to insure agglutination by fertilizin. However, even granting this unlikely possibility it is clear from the experiments with dermal secretion-fertilizin mixtures that the dermal secretion destroys the agglutinating action of fertilizin. In the first series of these experiments non-agglutinating mixtures were heated sufficiently to destroy the inhibiting agent but not the fertilizin. Such heated mixtures failed to agglutinate sperm and to inhibit agglutination. In the second series sperm were washed from unheated inhibitor-fertilizin mixtures containing sufficient fertilizin to block the sperm surface. These washed sperm agglutinated on addition of fertilizin. These experiments show that the dermal secretion of *Arbacia* can inhibit agglutination by inactivation of the agglutinin fertilizin. This inactivation is not simply a conversion of the fertilizin molecule to a form similar to the univalent fertilizin produced by some other agent (see Metz, 1957b, for review). The inhibiting agent in the dermal secretion inactivates or blocks the combining sites of the fertilizin that react with the sperm surface in agglutination.

In view of this action on fertilizin it is surprising that the dermal secretion has no visible effect on the sea urchin egg jelly since this jelly consists of undissolved fertilizin. It is even more surprising that eggs washed from dermal secretion will agglutinate with antifertilizin, since the agent in the dermal secretion inactivates the combining sites of fertilizin. These unexpected observations are explained by experiments showing that the combining sites of fertilizin are not inactivated by dermal secretion when the fertilizin is in the gel form. Possibly the inhibiting agent fails to diffuse through the egg jelly. However, it seems more likely that the specific combining sites are inaccessible to the dermal secretion because they are blocked by cross linkages in the gel structure. This concept has been offered as a possible explanation of passage of sperm through the egg jelly without saturation by fertilizin (Tyler, 1941). It is also consistent with the observation that fertilizin in solution and in the gel form differs in staining properties with metachromatic dyes (Monroy *et al.,* 1954). Even with this explanation one difficulty remains. If the combining sites of fertilizin in the gel form are not available for reaction with dermal secretion, then they might reasonably be expected to be unavailable to antifertilizin as well. This difficulty is resolved by assuming that egg jelly precipitation results from combination of antifertilizin with a part of the fertilizin other than the specific combining sites involved in sperm agglutination.

Finally, consideration of the effect of dermal secretion on the egg jelly precipitating action of antifertilizin leads to other interesting implications. In the presence of dermal secretion antifertilizin fails to precipitate egg jellies. This inhibition results from a second, heat-stable agent in the dermal secretion which acts upon antifertilizin. This complicates the problem further because the first experiments performed in this study show that the dermal secretion does not irreversibly inactivate the combining sites of the sperm surface antifertilizin. Therefore, the heat-stable inhibitor must inactivate egg agglutinating antifertilizin preparations by combination with some other part of the molecule. A final possibility that is not excluded is that the "antifertilizin" extracted from sperm is not related to the sperm surface material that combines with fertilizin in the sperm agglutination reaction. The "antifertilizin" may be a fortuitous product of the extraction procedure which, like some other proteins, precipitates sea urchin egg jelly nonspecifically.

One other question requires consideration; namely, the relation of the inhibitors described above to inhibition of fertilization. Like the sperm agglutination inhibitor the fertilization inhibitor is heat-labile. This is consistent with the view that inhibition of agglutination and of fertilization result from action of the same agent. Further investigation of this relationship may reveal that the fertilization inhibiting action of dermal secretion results from inactivation of fertilizin.

Other agents are also known to inhibit fertilization in the sea urchin. Notable among these are the preparations from the brown alga, *Fucus,* studied by Runnström and co-workers. Two inhibitory agents are recognized. One is a phenolic substance and has not been studied in great detail (Wicklund, 1954). The other, named "fertilization inhibitor (Fucus)," is heat-stable, increases motility of sperm (Wicklund, 1954) but does not inhibit fertilizin agglutination of sperm (Runnström and Hagstrom, 1955). Clearly this agent has different properties than the dermal secretion and the two preparations probably act in different fashion to inhibit fertilization. Indeed, they may very well block different steps in the initial stages of fertilization. Accordingly, a detailed analysis of the site and mechanism of action of these and other inhibitors might provide interesting information concerning the sequence of events in the initial stages of fertilization.

SUMMARY

1. As demonstrated previously by Oshima (1921) and Pequegnat (1948) *Arbacia* release a yellowish secretion upon appropriate stimulation.

2. This dermal secretion increases the motility and oxygen consumption of sperm. The effect is not short lived and does not appear to depend upon a metal binding action.

3. The dermal secretion inhibits fertilizin agglutination of sperm. This action results from an inactivation of the specific combining sites of fertilizin. The dermal secretion does not act upon the sperm surface to inhibit agglutination. The agent in dermal secretion that inactivates fertilizin is destroyed by heating to 100° C. and fails to diffuse through cellophane.

4. The dermal secretion has no visible action on the *Arbacia* egg or egg jelly.

5. The dermal secretion inhibits antifertilizin precipitation of intact egg jellies. This action depends upon a heat-stable agent which precipitates antifertilizin.

6. Some properties of the dermal secretion are described. The material contains at least three distinct antigens.

7. The results are in agreement with the view that fertilizin is essential for fertilization.

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BODY TEMPERATURES IN SOME AUSTRALIAN MAMMALS.[1]
I. CHIROPTERA

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The Microchiroptera are a group of mammals which show unique thermoregulatory behavior since they alone exhibit a daily reversal between homeothermic and poikilothermic conditions. Their poikilothermic nature is without question since their body temperature (T_B) often closely follows the ambient temperature (T_A) (Hock, 1951; Eisentraut, 1956a). However, some question has been raised as to the nature of their homeothermism at other times by the suggestion that they must be physically active in order to maintain their temperature; the implication being that the elevation is a passive concomitant of the heat produced during exercise, analogous perhaps to the warming of a bumblebee or a tarpon, and thus may not represent positive regulation. In the present study the relation between activity and body temperature is examined in one microchiropteran to throw light on this question.

The situation in the Megachiroptera is also in some question. Although a limited number of measurements (about a dozen) have suggested considerable regulation in this group of larger bats (Burbank and Young, 1934; Eisentraut, 1938) the statement has still been made recently, that all bats exhibit a lack of thermoregulatory control when they become inactive. The following observations on a representative of this group will allow us to compare it more closely in this regard both to other mammals and to the suborder of smaller bats.

MATERIAL AND METHODS

The Microchiroptera were represented by the bent-wing bat, *Miniopterus blepotis.* This genus of simple-nosed bats (Vespertilionidae) is distinguished by the extended terminal joint of the longest digit which is bent up against the upper part of the wing during rest, and by the tail length which is equal to the head and body. This species ranges through most of Australia and is known for its communal, cave-dwelling habit (Troughton, 1941). These individuals were between our little brown and big brown bats in size with a weight range from 6 to 12 grams. The animals were taken from a colony living in a cave formed by fallen slabs of rock in a small stream about 50 miles north of Brisbane. Its depth was about 30 feet and access was through the falling water, although other smaller openings were available to the bats. The animals were closely packed, hanging from the ceiling over an area of several square yards. They became active quite quickly on disturbance since the ambient temperature was not low in this first month of spring (September). A subsequent visit in the fall (March) showed only a few scattered individuals, but whether this was a seasonal change or a result of the previous disturbance is not known. We are greatly indebted to Dr. Peter Ulrich for his assistance in locating and procuring these *Miniopterus*.

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The megachiropteran studied was the grey-headed fruit bat, *Pteropus poliocephalus,* a representative of the single Australian genus, which was kindly loaned us from his collection by Mr. David Flea of West Burleigh. This individual, a

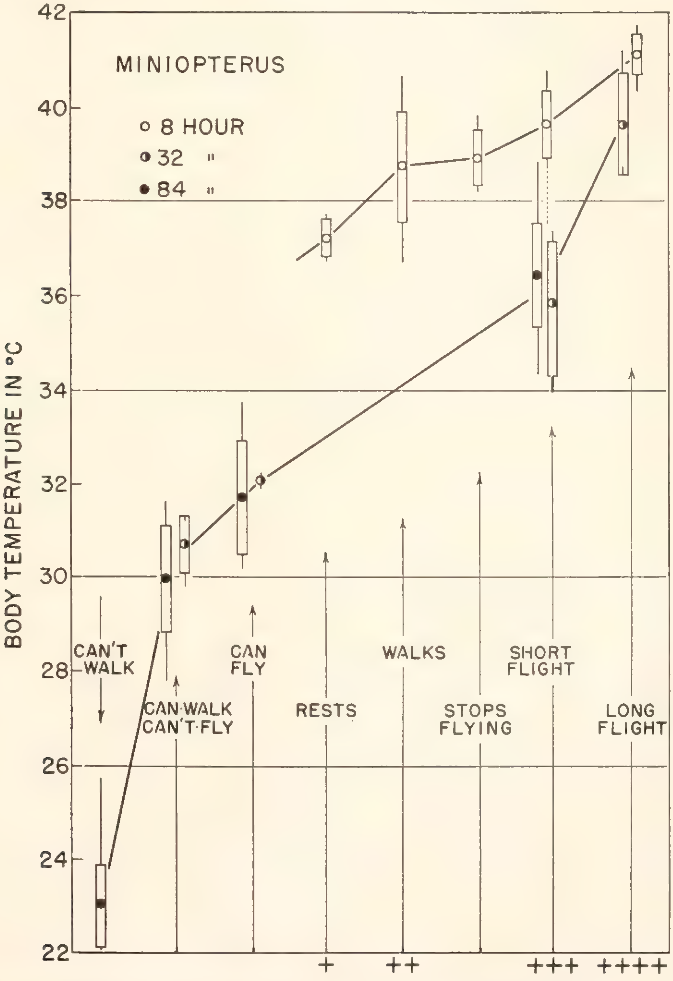

FIGURE 1. Relation between body temperature and activity in *Miniopterus.* Symbols indicate hours after capture. Ambient temperature, 19–21°.

250-gram male, had been well adjusted to captivity but following transfer it showed some progressive readjustment during the week it was studied. This species, which like its fellows feeds on fruit and eucalyptus blossoms of various sorts, is found in central-coastal, eastern Australia. It has been distinguished from the three other mainland species by its longer coat on body and legs which give it a furry appearance (Troughton, 1941). Specimens up to a kilogram in weight and with a 4-foot wing spread have been reported.

Body temperature measurements were made with iron-constantan thermocouples and a Leeds and Northrup recording potentiometer. The *Miniopterus* were flown in a large room, which, however, did not provide sufficient space to fly the *Pteropus.* One set of observations was carried out on the former animals at night within 8 hours of capture. The group was then stored in a refrigerator at 5–10° and subsequent observations were made one day and three days later. Metabolic measurements of oxygen consumption utilized a manometric apparatus (Morrison, 1951) and were carried out with the assistance of Mr. Len Morris and Miss Nancy Harvey.

RESULTS

Miniopterus

The relation between activity and body temperature is summarized in Figure 1 in animals observed within 8 hours of capture. Activity is expressed as resting

TABLE I

Body temperature and activity in Miniopterus

Activity level	Hours after capture	Mean ± S.D.	No.	Range
Cannot walk	84	22.7 ± 0.36	7	22.1–23.3
		23.0	8	22.1–25.7
Can walk	84	30.0 ± 1.15	6	27.8–31.6
Cannot fly	32	30.7 ± 0.59	5	29.8–31.2
Can fly	84	31.7 ± 1.24	9	30.2–33.7
	32	32.0 ± 0.10	4	31.9–32.2
Resting after activity	8	37.2 ± 0.40	6	36.7–37.7
Walking	8	38.7 ± 1.16	16	36.7–40.6
Stop flying	8	38.9 ± 0.61	5	38.2–39.8
Short flight	84	36.4 ± 1.10	8	34.3–38.8
	32	35.8 ± 1.55	3	33.9–37.1
		39.6 ± 0.72	8	38.7–40.7
		39.2 ± 1.08	11	37.4–40.7
Long flight	32	39.6 ± 1.07	10	38.7–41.2
	8	41.1 ± 0.45	13	40.3–41.7
	8	39.1 ± 1.0	49	36.7–41.7

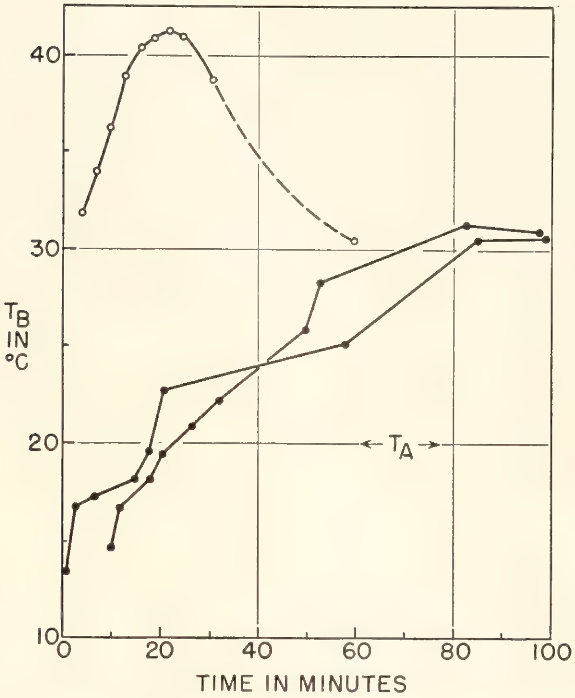

FIGURE 2. Course of temperature changes during awakening and extended flight. $T_A = 20°$. Upper solid curve indicates flight at *ca.* 600 ft./min., followed by rest.

(1+), walking or stops flying (2+), short flight (3+) and long flight (4+). These symbols follow a scale of relative activity which has been used with other mammals (Morrison and Ryser, 1959), and ranges from sleep (0+), through awake (1+), moving (2+), active (3+) and very active (4+). A condition of sleep (0 +) was not seen in any of these initial observations which were carried out in the evening when the bats are normally active. Further, these animals were undoubtedly disturbed by their recent capture and travel. Actually, the majority of these animals were actively walking or flying, so that only a few were in the "resting" state. However, the observed range of activity was as wide as in most animals since it is usually not possible to elicit maximum activity (4+).

A regular increase in body temperature with activity level is seen. Values given in Table I show a mean increase of 1.3° per activity grade, or an over-all increase of 3.9° (1 + → 4 +). These 49 values may be averaged to give a mean for the awake condition, although this is an artificial situation and so gives an artificial distribution of activity. This mean is 39.1° ± 1.00 (49), at a mean activity of 2.6 + and with an over-all range of 5.0° from 36.7–41.7°.

The values taken 32 and 84 hours after capture are similar, but they are quite distinct from the earlier observations. After these animals were taken from the refrigerator and warmed to room temperature, many were loath to engage in any activity. Below a body temperature of 26° the bats could not walk; at 28–31.5°

the bats could fly when launched, sometimes with difficulty, but would not take off spontaneously. They would bite.

Some of these temperatures were transient values during warming, but others represented a maintained level in an inactive animal (0 + activity level). Thus, for example, one bat (A) which was followed during warming rose to 22.7° (+ 9.3°) in 20 minutes, and to 30.3° (+ 12.2°) in another hour, but only to 30.6° (+ 0.3°) in another 15-minute period (Fig. 2). Another, warmed to 22.1° (+ 7.5°) in 20 minutes, and to 28.2° (+ 6.1°) in a second 20-minute period. After a third period the value rose to 31.2° (+ 3.0°), but with a fourth period a slight fall to 30.8° (− 0.4°) was observed. Accordingly, these quiet animals are maintaining their body temperatures well above the ambient temperature of 20° ($\Delta T = +\ 10°$) but at the same time, greatly below the level of more active states; 10° less than the actively flying animal.

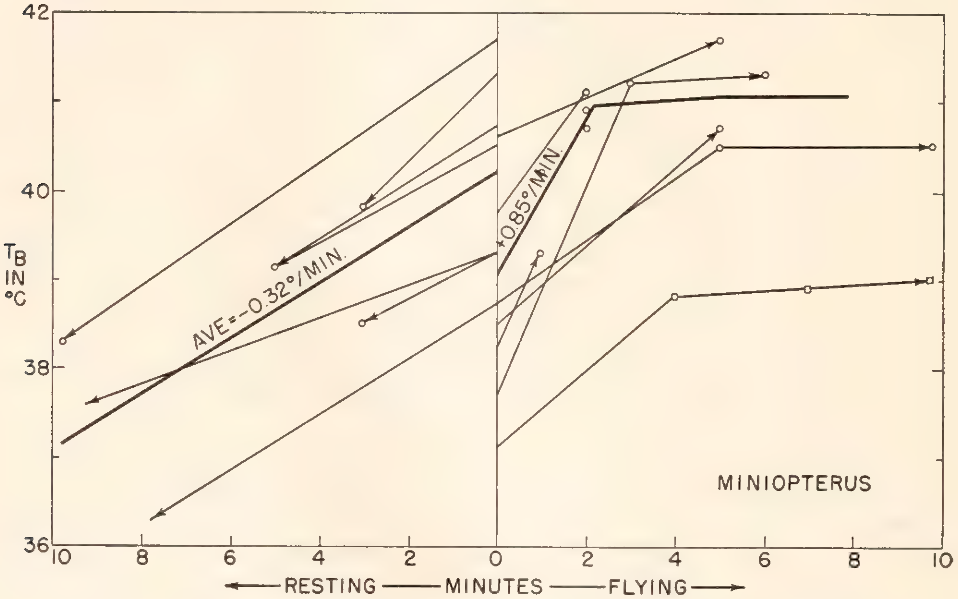

FIGURE 3. Changes in body temperature following the initiation or completion of flight in *Miniopterus.*

Some animals become active spontaneously and others were forced to be active. Under these conditions shorter flights were made and lower body temperatures were recorded than before (36° vs. 39°). The animals which made prolonged flights averaged 3° warmer, equivalent to the "short flight" (3+) condition in the fresh bats, but still 1.5° below the "long flight" (4+) level in fresh bats.

Figure 3 presents successive measurements on individuals to show temperature change in active bats. During flights an average increase of 0.85°/min. was seen for the first two or three minutes. After this the temperature leveled out abruptly, showing a quite positive regulation at this higher temperature. The decline in body temperature following flight was more gradual (− 0.32°/min.) but could continue for as long as 10 minutes without leveling off at a lower value. Figure 2

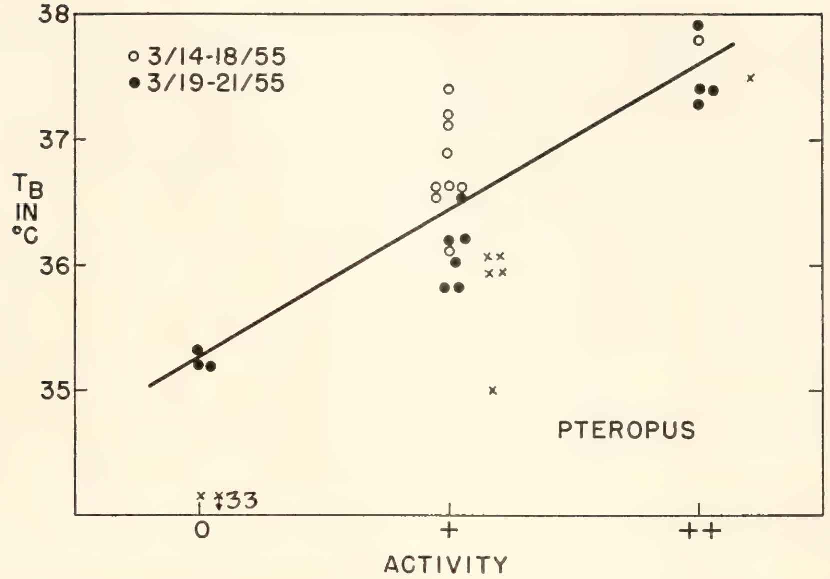

FIGURE 4. Relation between activity and body temperature in *Pteropus*. $T_A = 25°$. Average ± S.D. (⋇): 0 (asleep) = 35.2 ± 0.05 (3); + (awake, not moving) = 36.5° ± 0.49 (15); open circles only = 36.8 ± 0.39 (9); closed circles only = 36.1 ± 0.27 (6); ++ (moving around or feeding) = 37.5 ± 0.25 (5). Slope of curve = 1.2°/+. Crosses show values of Burbank and Young (1934) for *P. geddei*, *P. giganteus* and *P. cotinus* at $T_A = 19°$.

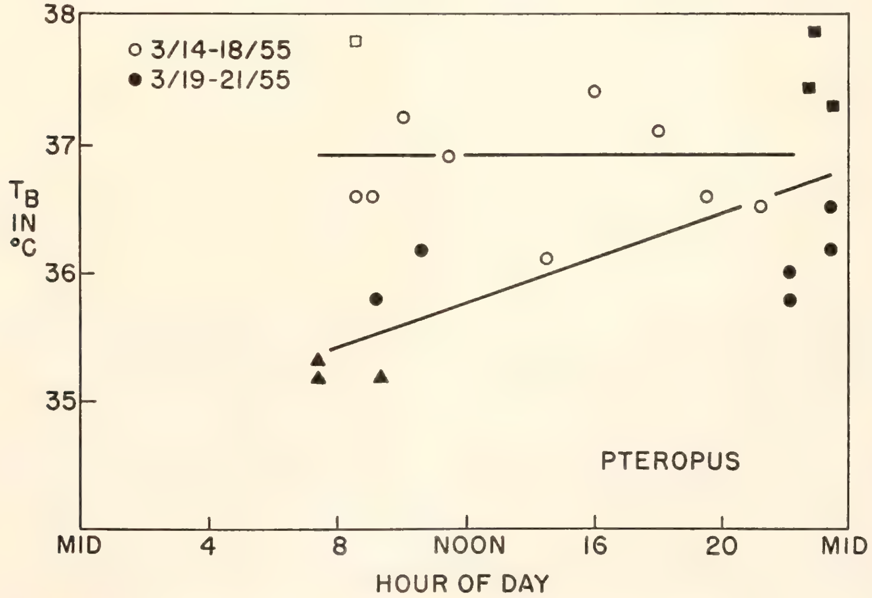

FIGURE 5. Body temperature in *Pteropus* as a function of the hour of day. $T_A = 25°$. Average values for solid symbols: 7 → 10 = 35.5° (5); 22 → 24 = 36.7° (7). Open symbols: 8 → 14 = 36.9° (6); 16 → 22 = 36.9° (4). △ = 0, ⊙ = 1+, ⊡ = 2+ activity.

shows a value 40 minutes after stopping activity which has returned to the level of quiet, awake animals.

These changes in body temperature may be used to estimate heat production during warming. Thus, a maximum increase of 1.03°/min. (11.5 → 23.8° in 12 minutes) was observed in one individual. This is equivalent to 0.85 cal. g.$^{-1}$min.$^{-1}$ or an oxygen consumption of 10.7 cc. g.$^{-1}$hr.$^{-1}$ (other cases (24.6 → 32.2° in 10 minutes; 22.2 → 28° in 12 minutes; 22 → 28.3° in 14 minutes) were equivalent to metabolic levels of 7.9, 5.0 and 4.7 cc. O_2 g.$^{-1}$hr.$^{-1}$.) Of course, the first estimate with body temperature below ambient temperature was high because of the heat flow into the animal, and the latter three were low for the reverse reason. An average maximum between these is equivalent to 9 cc. g.$^{-1}$hr.$^{-1}$. This is a substantial but not unreasonable rate for such a small animal. But when it is recalled that the body temperature was only 15–25°, it is much more striking. Even with

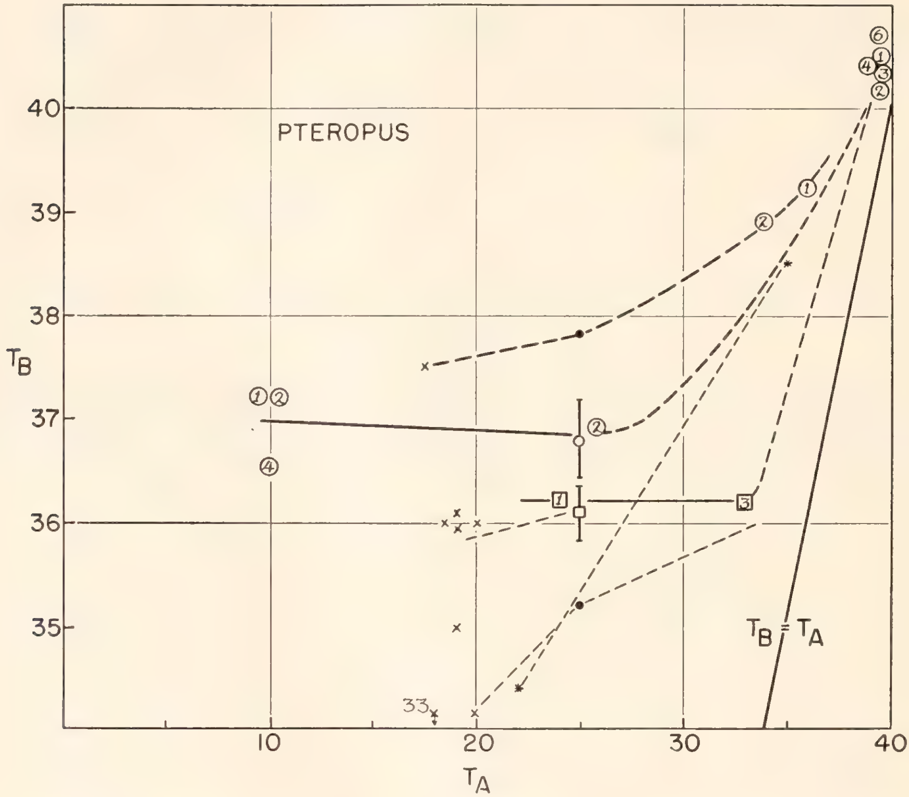

FIGURE 6. Body temperature in *Pteropus* after exposure at various ambient temperatures. Values indicate hours of exposure. Circles, earlier data (3/14–18) and squares, later data (3/19–21) at 1+ activity. Top and bottom curves are for 2+ and 0+ activity, respectively. Crosses show values of Burbank and Young (1934). Stars and dotted line show range of ambient and body temperatures reported by Eisentraut (1938) for *Rousettus angolensis*.

a modest Q_{10} of 2.0, this would be equivalent to a rate of some 36 cc. g.$^{-1}$hr.$^{-1}$ at 40° or greater than the maximum observed even in the smallest mammal, the long-tailed shrew (Morrison *et al.*, 1953, 1959).

Pteropus

Data relating activity to body temperature in this form are presented in Figure 4. Because of its size, it was not possible to fly it, and thus activity was limited to moving about its cage. However, three observations during sleep (0 activity) were made. These animals had closed eyes and were distinctly less responsive on handling. The average increment was 1.2°/+. The average temperature was $36.5 \pm 0.77°$ (23) at a mean activity of 2.1 +.

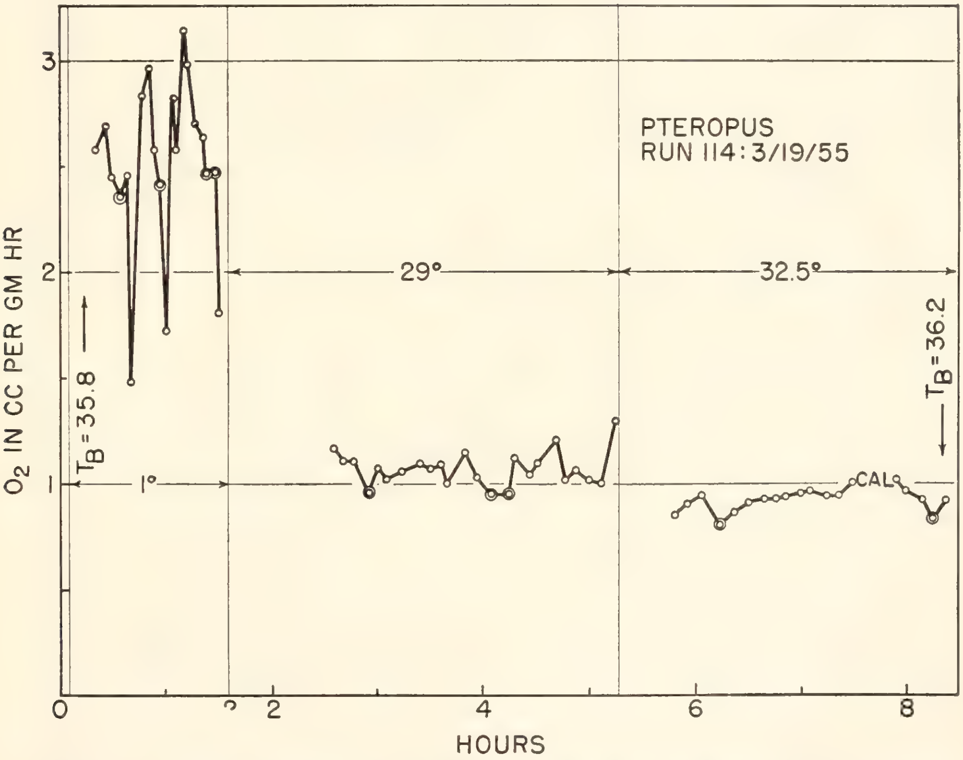

FIGURE 7. Oxygen consumption in *Pteropus* at three ambient temperatures. Representative experiment showing variation, particularly in the form of short reductions from the average level at $T_A = 1°$.

Temperature values for *Pteropus* are plotted against the hour of day in Figure 5. During the first four days no value less than 36° was observed and no daily cycle was seen. During the last three days, after the animal had settled down, there was a definite diurnal difference with the day-time values (7–10) averaging 35.5° and the night-time values (22–24) averaging 36.7°.

The influence of ambient temperature on body temperature in *Pteropus* is shown in Figure 6. Exposures of up to 4 hours at 10° had little effect on the body temperature. At an ambient temperature of 40° the body temperature was maintained roughly constant through a 6-hour exposure but the level was elevated to between 40 and 41°. The average value of 40.5° provided a temperature differential of only 0.5° for the dissipation of body heat. However, this small differential was apparently adequate because of the very large surface available in the wings, and the ceaseless fanning activity during this heat stress (Robinson and Morrison, 1957). During the later period after the bat had settled down presumably with a lower resting metabolic rate, the upper limit of thermal neutrality appears to have been substantially raised.

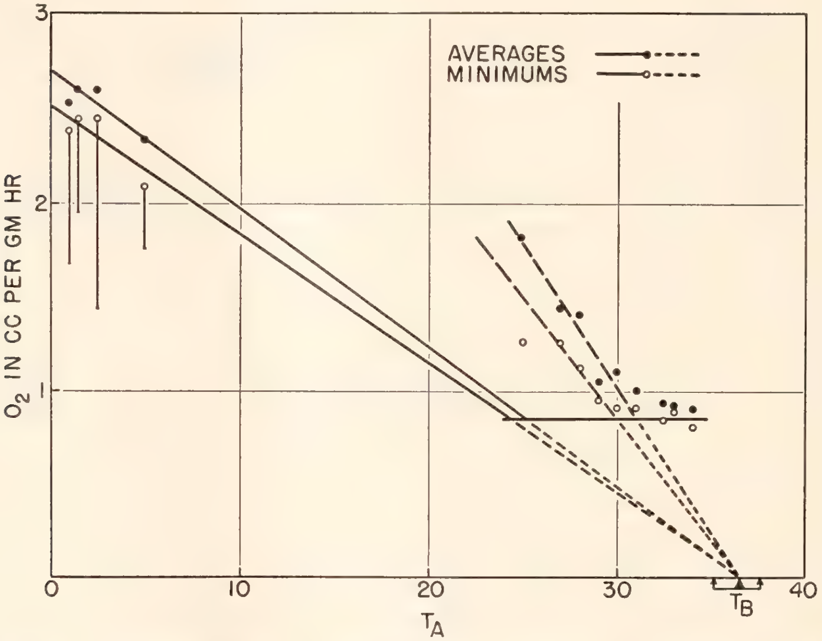

FIGURE 8. Oxygen consumption in *Pteropus* at various ambient temperatures. Minimum and average values from three runs (#100, 105, 114). Bottom pair of curves shows conductance at low temperature; top pair, at room temperature.

The metabolic response to cold exposure measured as oxygen consumption is illustrated in Figure 7. The measurements at higher temperatures (28–34°) were extremely regular, reflecting the behavior of the animal which hung quietly. Indeed, for all measurements the averages for each temperature period (12) were only 10% higher than the minimum values, and the maximum values were only 32% higher. Minimum and average values are plotted in Figure 8 against ambient temperature. There is some question as to which of these most accurately describes the minimum conductance at each temperature. The average value may be too high since extra heat (*i.e.*, above maintenance) is dissipated during movement and activity. On the other hand, if there are fluctuations in body temperature a minimum value may represent not equilibrium but a negative heat balance. Such a situation appears in the series at $T_A = 1°$ in Figure 7, which is characterized by several periods of brief duration and much lower rate. We interpret these as inadequate heat production. The oxygen deficit and durations (*ca.* 1 cc. $g.^{-1}hr.^{-1}$ and 3 min.) correspond to a temperature change of 0.3°, and it may be noted that each of these low periods is followed by a period of increased, presumably compensatory, activity. We have previously observed this type of response (a sharp, brief fall) only in hibernators entering hibernation. These low values are excluded in our estimate of conductance but their values are indicated in Figure 8 along with the average and minimum values. We feel that the latter is preferable in calculating minimum conductance, but in this species the difference is only 10%.

In the simplest model homeotherm the metabolism below thermal neutrality is represented by a single curve, $M = C\ (T_B - T_A)$. This is not possible in the present case which requires separate curves for the cold and for room temperatures. This is not an uncommon situation and simply means that at moderate temperatures the animal does not make use of its maximum insulative potential. In this instance, the higher conductance at 25–30° (0.13 cc. O_2 $g.^{-1}hr.^{-1}{}^{\circ}C.^{-1}$) is about twice the minimum value measured at 0–5° (0.068 cc. O_2 $g.^{-1}hr.^{-1}{}^{\circ}C.^{-1}$).

DISCUSSION

The behavior of *Miniopterus* shows that when awake these animals can act as homeotherms with a regulated body temperature comparable to other mammals. On the night following capture the group was all awake and, although some were quite inactive, no temperature less than 36.7° (out of 49 values) was observed. While the level of the temperature varied with the level of activity, this is a normal feature in homeotherms. The activity increment of 1.3°/+ or 3.9° from "awake" to "very active" is comparable to that in non-hibernating mammals; and indeed is less than that seen in some. The average temperature of 39.1° is more than a degree above the average for mammals (Morrison and Ryser, 1952). Although the mean activity represented by these values is high (2.6 +), the value corrected to a level of 1 +, 37.0°, is still a reasonable one. Although 1° below the mean for "all" mammals it is within one standard deviation (for species) and is close to mean values for other hibernators in the awake condition. And it is almost identical to that of *Sorex*, a small representative of another primitive group of mammals, the insectivores (Morrison *et al.,* 1953, 1959).

The observed values are also in accord with values of 37.8 and 38.2° made on two "fully awake" but not flying individuals of a related species, *M. inflatus* (Eisentraut, 1956b), since the latter lie within our range for walking animals. This range, 36.7–40.6°, is slightly higher, but quite comparable to those for 5 species of temperate bats (limits, 34.4–40.8°) cited by Eisentraut (1938). But it is distinctly above the range given by him for 5 tropical species from Africa (limits 28–39.2°). Swanson and Evans (1936) report values of 36.7 and 36.1° for aroused *Eptesicus* in winter.

Values for animals following extended flight were the most uniform of any activity level (41.1 ± 0.45° (13)) and this may reflect the more positive thermoregulation in response to the excess heat of flying, using the very effective dissipating potential of the wings. Burbank and Young (1934) reported a maximum temperature of 41.0° after flight in the horseshoe bat (*Rhinolophus*). In *Myotis yumanensis,* Reeder and Cowles (1951) observed temperatures of 39.0–40.3 after flight and reported that vasodilation of the wings took place at a body temperature of about 40°. This species also appears to be similar in its critical temperature for flight, 31°, which represents the dividing point for *Miniopterus.* Burbank and Young also note 30° as the minimum temperature for flight in *Rhinolophus,* and 30–33° as the temperature for initiation of flight in the noctule bat, *Nyctalus noctula.* Minimum temperatures for flight in hibernating birds are also somewhat above 30°. Miller (1950) reported a temperature of 34.0° after a short flight in a poor-will (*Phalaenoptilus*). And Marshall (1955) described an awakening captive individual that could not fly at a body temperature of 34.4°.

Following refrigeration at 10° some of the animals appeared in a different condition. On transfer into the room they warmed to a level well above room temperature (31°), but much less than before even though some walked. Eisentraut (1956b) reported the *M. inflatus* did not warm above 30° after refrigeration. Other *Miniopterus* in the present study flew and warmed to a higher level, though still less than following short flights in fresh animals. However, with extended (forced) flights, almost as high values were noted. But after activity the body temperature again fell to near 30°. This approach to the same level from above as from below (Fig. 2) provides rather convincing evidence of a maintained level and it appears significant that this level is such as to just permit flight. This suggests that the lower temperatures did not result from any energetic deficiency, but possibly from a less disturbed condition than on the day of capture. Pearson (1947) in metabolic measurements, observed a case of continuously elevated metabolism during the day in a specimen of *Eptesicus* which was disturbed for some reason. It may be that bats can maintain different levels of temperature, without exercise, in relation to different excitatory levels.

In this regard it should be noted that although bats have been considered unique in their daily transformation into poikilotherms, observations from another phase of this study have revealed essentially this same behavior in one of the Dasyurid marsupials, *Chaetocercus.* During the day this animal's temperature may fall close to the ambient temperature even though this is as high as 28°. A related, but not so pronounced effect has also been observed in the deer mouse (*Peromyscus leucopus*) in which certain individuals cool substantially beyond the ordinary range of variation during enforced inactivity even at moderate temperatures (Morrison and Ryser, 1959).

Slonim (1952) describes studies of body temperature and metabolism in relation to ambient temperature in four microchiropterans. Two species (*Miniopterus schreibersi* and *Myotis Myotis*) showed clear temperature regulation between 13 and 24° with a maintained temperature level and a strong metabolic response of 17–20%/° C. Two species of horseshoe bats (*Rhynolophus ferrum equinum* and *R. hipposideros*) showed limited, irregular response (at 20–25°). However these latter observations were made in March and in the fall, while the first mentioned species were studied in May and June. In all these experiments (10 values) the maintained body temperatures ranged between 29 and 33°. It may be of significance that this level of regulation (31–33°) is also that found in our most primitive homeotherm, the echidna (Wordlaw, 1915).

The rates of rewarming were comparable to the maximum rates observed in the same genus (1.28, 0.93°/min.) by Eisentraut (1956b) and in *Myotis* (1.33°/min.) by Reeder and Cowles (1951). But our maximum rate of warming during flight (*ca.* 1.0°/min.) was substantially less than a value for *Myotis.*

Pteropus

The influence of activity on body temperature in *Pteropus* appears quite similar to *Miniopterus* with an activity increment of 1.2°/+, although the observed range was more limited. If we correct the mean body temperature of 36.5° (2.1 +) to a standard activity of 1+, we have a value of 35.2°. This is definitely lower than in *Miniopterus* and the average for mammals, but it is quite comparable to values for two larger insectivores, the mole and the hedgehog, 35.5 and 35.8° (Morrison and Ryser, 1952). Similarly, the variability of the temperature ($\sigma = 0.77°$) is reasonable. The range, 35.2–37.9°, is somewhat less than that of 34.4–38.6° ($T_A = 22$–$35°$) reported in an African form, *Rousettus angolensis* by Eisentraut (1938), but the midpoint of this range (36.5°) falls just at our mean value. The other observations on megachiropterans are by Burbank and Young (1934). They found an average of 35.9 ± 1.4° (8) in measurements on three species of *Pteropus.* Excluding one value for an active and two for sleeping individuals gave an average of 35.8° or within the σ of our second group of values. Their single value on an actual individual (37.5°) checks well with our value at 2 + activity (37.6°), but two values on a "large male hanging quietly" were well below our range of values (33.0 and 34.0°). Possibly the lower ambient temperature (18°) in these studies was only of influence at 0 + activity. Under these conditions they noted a response of shivering and a maintenance of body temperature between 33.0 and 37.5°. This is considerably more variation than we observed in our specimen, which maintained a body temperature of 36.5 even after a 4-hour exposure at a lower temperature of 10°. But if we limit Burbank and Young's values to the four at 1 + activity (neither active nor asleep) corresponding to our specimen in the cold, a close correspondence is seen (36.8 *vs.* 36.5°).

It might be suggested that conditions of captivity prevented the observation of temperature lability which still might be present in nature. This indeed appeared to be the case in several specimens of *Chaetocercus* referred to above. While this suggestion cannot be disposed of completely in *Pteropus,* what evidence there is appears against it. Field observations show that these animals are not sluggish and lethargic during the day as are smaller bats. Rather, we found them alert and able to fly off at once when their roosting place is approached or their roosting tree disturbed, even at midday.

Metabolic measurements confirm observations on body temperature in the cold. *Pteropus* can substantially increase its heat production, the average minimum value at 0° being 2.5 cc. O_2 g.$^{-1}$hr.$^{-1}$ or 3 times the B.M.R. This rate corresponds to a thermal conductance, *C,* of 0.068 cc. O_2 g.$^{-1}$hr.$^{-1}$° C.$^{-1}$. This minimum value is close to that observed in similar measurements on small mammals from Wisconsin (Morrison and Ryser, 1951). This series could be described as $C = 0.95\ W^{-.48}$ which function has a value of 0.071 cc. O_2 g.$^{-1}$hr.$^{-1}$° C.$^{-1}$ at 250 grams. Accordingly, this subtropical megachiropteran has an insulation quite comparable to temperate species. This contrasts to the tropical mammals examined by Scholander *et al.*

(1949) which had inferior insulation as compared to Arctic forms. We have previously observed less effective regulation in several Central American species than that shown here by *Pteropus* (Morrison, 1946). The minimum temperature at Brisbane approaches, but does not go below 0°, so these bats had the capacity to regulate at any time during the year.

The "slips" in metabolism at low temperatures below the maintenance level (Figs. 7 and 8) might be interpreted as incipient hibernation since we have seen this phenomenon only in animals entering hibernation. However, there is nothing to confirm this view, and an alternative suggestion relates to short rests between bouts of shivering. Such a phenomenon has been observed in the dog with rests of one to fifteen minutes and a concomitant reduction of metabolism to the basal level (Hammel and Hardy, 1957).

A final point of interest relates to the basal metabolic level. The observed mean value of 0.84 cc. O_2 $g.^{-1}hr.^{-1}$ compared closely with the "standard" value for a 250-gram mammal, $3.8\ W^{-.27} = 0.87$ cc. O_2 $g.^{-1}hr.^{-1}$. Or conversely we may describe the metabolic rate in *Pteropus* as $3.7\ W^{-.27}$ cc. O_2 $g.^{-1}hr.^{-1}$. There are no data in the literature with which to directly compare this value. Hock (1951) reports a value of 3.0 cc. O_2 $g.^{-1}hr.^{-1}$ in *Myotis* at $T_A = 37°$ or about $5\ W^{-.27}$ cc. O_2 $g.^{-1}hr.^{-1}$. But although the body temperature probably lay between 37 and 38°, these measurements made over one-hour periods undoubtedly included some muscular activity and cannot be taken as minimum values. Accordingly, in the matter of the B.M.R. as well, the Chiroptera appear to be closely comparable to other mammals.

CONCLUSIONS

In summary we may conclude from these studies and other data that the Megachiroptera (*Pteropus*) are not to be distinguished from other mammals on the basis of thermoregulation as are the Microchiroptera. Their temperature is quite typical in regard to variability, activity response and cold response; and its level, although somewhat lower than average, is still higher than some other mammals of comparable size such as the insectivores.

Although the Microchiroptera (*Miniopterus*) are well developed hibernators which can become poikilothermic when circumstances demand, when awake they may show thermoregulatory behavior which is comparable to other mammals in regard to variability, activity response and mean level. But they may also show an intermediate maintained level, neither poikilothermic nor homeothermic in the usual sense.

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STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] III. THE EFFECT OF COMBINED RADIATION TREATMENTS ON PHOSPHORYLATION

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Previous work has established that particulate-linked, oxidative phosphorylation is very sensitive to ionizing radiation. This is true whether the radiation is delivered to the whole organism (van Bekkum and Vos, 1955) or to the isolated particulates (Yost and Robson, 1957). These findings suggest that one of the principal modes of action of radiation is the destruction of the cells' ability to carry on their "energy metabolism." Interference with oxidative phosphorylation would be expected to have profound effects upon all reactions of the cell which are dependent upon a supply of ATP (the synthesis of proteins and nucleic acids being the most obvious). This suggests that some of the observed damage to the chromosomes resulting from radiation might be the result of extra-nuclear damage to the phosphorylating mechanism, which suggestion is supported by the observation of many investigators (see Wolff and Luippold, 1955) that the number of chromosomal aberrations recovered after radiation treatment is altered by known inhibitors of oxidative phosphorylation.

The radiation literature contains many references to the effects of combined radiations in producing alterations in chromosomal aberration (Giles, 1954). It has been shown that pretreatment with ultraviolet radiation will cause a *decrease* in the number of translocations recovered after exposure to ionizing radiations (Swanson, 1943). On the other hand, pretreatment with near infrared radiation causes an increase in the recovered aberrations resulting from exposure to ionizing radiations (Swanson, 1949). To date, no completely satisfactory explanation has been advanced for these results. Explanations of complimentary or antagonistic action by radiations used in combination are complicated by the duration of these effects. In the case of infrared treatment, there may be a time lapse of at least 96 hours between the application of the infrared and the x-rays with no decrease in effectiveness. Thus, the condition induced is stable, although it may be altered by drastic environmental changes (Swanson and Yost, 1951). However, when the data from combined radiation studies are taken together with the data on the effects of combined radiation and chemical treatments (Kihlman, Merz and Swanson, 1957), an hypothesis may be formulated. It is possible that the various combined treatment effects are the result of activation or inactivation (depending upon the radiations or chemicals used) on oxidative phosphorylation.

The data presented in this paper are the result of studies designed to test the hypothesis that the observed effects of combined radiation treatments are the result of interference with the normal phosphorylation pathways. The effect of ultraviolet radiation alone and in combination with gamma radiation, and the effect of infrared radiation alone and in combination with gamma and ultraviolet radiations was studied. Isolated particulates from rat liver were chosen since the effects of gamma radiation and some ultraviolet effects were already known (Yost and Robson, 1957; Yost, Robson and Spiegelman, 1956).

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Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where much of the blood is washed free. The liver was weighed and pressed through a bronze screen to remove connective tissue. The resulting mash was then suspended in 50 ml. of cold 8.5% sucrose containing 0.005 *M* disodium versenate and homogenized in a glass homogenizer. The mitochondria were then separated from the rest of the homogenate by differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept. The final centrifugate was re-suspended in 1 ml. 8.5% sucrose per gram of original liver. All dilutions were made from this stock solution.

For treatment with gamma radiation the preparation was diluted 1 in 20 with cold distilled water. This diluted suspension was radiated in a 25-ml. "Lusteroid" centrifuge tube in the beam of a 440 curie Co^{60} source. The radiation was filtered by a half-inch lucite shield to remove the beta radiation. The intensity of radiation was 1000 r per minute. The controls were treated in the same manner, with the exception of the exposure to radiation. After radiation, control and treated suspensions were centrifuged, and the mitochondria re-suspended in one ml. This final suspension was assayed for phosphorylation.

For treatment with ultraviolet radiation the preparation was not diluted. Two and five-tenths ml. were placed in a quartz flask rotated in the ultraviolet beam by a slow speed stirring motor; 2.5 ml. were kept in a Pyrex tube in the cold as a control. During the period of irradiation, the quartz flask was partially submerged in an ice-water bath maintaining a temperature under 5° C. This flask was kept 11.5 cm. from a germicidal lamp for all radiations. The intensity of the radiation was 5500 $ergs/cm^2/sec$. For most studies the radiations were filtered by a Corning No. 7910 filter which transmits principally those wave-lengths above 2400 Å (Filter I). In one case, to test the effect of longer wave-lengths, a Corning No. 9700 filter (transmits principally above 2700 Å) was used (Filter II). To test the effect of the flask rotation on phosphorylation, experiments were run in which the mitochondria were treated in the same way that they would have been for ultraviolet treatment with the exception that the ultraviolet source was not turned on.

For treatment with infrared radiation, the preparation was not diluted. Four or five ml. of the suspension were placed in a small test tube which was rotated in the infrared beam by a slow stirring motor. Temperature changes were eliminated by radiating the suspension while immersed in a water bath cooled by a Blue-M cooling coil. The bath was maintained at 9° C. for all studies. Controls were kept in the rear of the water bath wrapped in aluminum foil to prevent exposure to radiation; in two cases they were rotated as were the treated. The infrared source was 6 inches from the center of the exposed test tube. All radiation passed through a Corning No. 2540 filter, the glass side of the water bath (window glass), and 1 cm. of water. This arrangement provides intense radiation in the region 7600 to 15,000 Å. The source of radiation was a 250-watt commercial heat lamp operated at 110 volts. The usual exposure was for three hours. The intensity of the source is not accurately determined, but this was capable of increasing the chromosome aberrations in Tradescantia by at least 100% when used in combination with gamma rays.

For studies of the effects of combined radiations, the procedures outlined above were followed. The controls were taken through all the steps with the exception of exposure to radiation.

Some attempts to reactivate the ultraviolet-damaged phosphorylation mechanism were made. In attempts to photoreactivate the system, the suspension was exposed to visible light from a 250-watt incandescent spot-light filtered to remove the red wave-lengths. This treatment was carried out at 10° C. in the same water bath used in the infrared studies, to prevent heat-inactivation of the system. Treatments were of 20 minutes duration. In attempts to reactivate the system by the addition of co-factors, the co-factor was added to the reaction flask prior to assay of phosphorylation and oxygen uptake. In one case, 0.1 ml. of 0.01 *M* DPN was added, and in the other, 0.1 ml. of 10^{-4} *M* FAD.

Estimation of phosphorylation was conducted by a modification of the method of Maley and Lardy (1954), using succinate as the substrate. The main compartment of the Warburg vessel contained: 0.3 ml. (30 μM) phosphate buffer (pH 7.4), 0.3 ml. 0.1 *M* sodium succinate, 0.8 ml. 8.5% sucrose, 0.1 ml. (0.3 μM) cytochrome-*c*, 0.3 ml. (6 μM) ATP (Schwartz, neutral), 0.1 ml. (30 μM) $MgSO_4$, 0.1 ml. (40 μM) KF, and 0.5 ml. of the mitochondrial suspension. The center well contained 0.1 ml. 5 N KOH, and the side arm held 0.5 ml. (10 mg.) of hexokinase (Pabst) dissolved in 0.15 *M* glucose. To assure that the initial pH of the reaction would be 7.4 or higher, the pH of some of the more acid reactants was adjusted with NaOH before addition to the flasks. Readings of the oxygen uptake were taken for 30 minutes, after which the reactions were stopped with TCA and the phosphate determined by the Lowry-Lopez method as presented by Glick (1949). All Warburg assays were carried out at 25° C. All assays were made in duplicate. All experiments were repeated at least three times.

RESULTS

The data presented in Table I show the effectiveness of ultraviolet radiation in the inactivation of oxidative phosphorylation. Although the phosphorylation mechanism is very labile and therefore shows variation from experiment to experiment and from assay to assay, it is clear that ultraviolet radiation inactivates phosphorylation rather easily. These data cannot be used to construct an inactivation curve because of the variability, but there is clear indication that the inactivation proceeds in a non-linear manner. It is also clear that the inactivation is not the result of either the time lag between the isolation of the mitochondria and the assay or the rotation in the quartz flask. Thirty minutes' rotation causes no loss of phosphorylation. Further, it can be seen that the filter which transmits principally those wave-lengths above 2700 Å is not effective in bringing about this change. It would appear that the effective wave-length lies somewhere between the minimum of Filter I and the minimum of Filter II, that is, between about 2200 and 2700 Å.

TABLE I

Inactivation of phosphorylation by ultraviolet radiation

Treatment*	No. runs	Phosphate uptake (μM)		% Decrease	% Decrease O_2
		Controls	Treated		
5 min. UV	3	11.1 ± 2.1	10.2 ± 2.6	8.1	6.2
10 min. UV	3	12.7 ± 1.7	11.1 ± 2.0	12.6	3.5
20 min. UV	14	12.8 ± 1.1	7.4 ± 1.5	42.2	11.8
30 min. UV	3	15.7 ± 1.4	6.4 ± 0.8	59.2	3.0
30 min. rotation	3	13.8 ± 1.7	13.8 ± 1.7	0.0	0.6
30 min. UV Filter I	3	10.2 ± 2.6	1.8 ± 1.5	82.4	21.7
30 min. UV Filter II	3	10.2 ± 3.1	11.7 ± 2.7	−14.7	−11.6

* Particulates undiluted; treated in rotating quartz flask. Filter I transmits principally above 2400 Å; Filter II transmits principally above 2700 Å.

One other thing should be noted. The inactivation of the phosphorylating mechanism bears no clear relationship to the inactivation of the cytochrome oxidase system. Oxygen uptake is essentially unaffected in many cases in which there is extreme damage to phosphorylation. This is in agreement with earlier findings with gamma radiation (Yost and Robson, 1957).

The data in Table II show that the inactivated phosphorylation mechanism cannot be easily revived. Addition of DPN to the system stimulates phosphorylation but does not overcome the effect of the ultraviolet. As a matter of fact, when the

TABLE II

Failure to reactivate ultraviolet-damaged phosphorylation

Treatment*	No. runs	Phosphate uptake (μM)	% Decrease	% Decrease O_2
Control	4	10.3 ± 1.1	—	—
Control + DPN	4	13.7 ± 0.9	−33.0	− 7.7
20 min. UV	4	6.7 ± 1.5	35.0	16.4
20 min. UV + DPN	4	7.3 ± 0.8	29.1	5.7
20 min. UV + DPN using DPN as control			46.7	11.7
Control	3	21.2 ± 0.7	—	—
Control + FAD	3	20.2 ± 1.0	4.7	1.5
20 min. UV	3	16.2 ± 1.4	23.6	−10.4
20 min. UV + FAD	3	14.2 ± 1.0	33.0	−19.0
Control	5	15.7 ± 1.2	—	—
20 min. visible	5	15.1 ± 1.9	3.8	2.1
20 min. UV	5	11.1 ± 2.2	29.3	1.8
20 min. UV + 20 min. visible	5	10.1 ± 2.5	35.7	2.0

* Particulates undiluted; treated in a rotating quartz flask. Ultraviolet filtered by Filter I; visible light filtered to remove ultraviolet and infrared. DPH (0.1 ml. of 0.01 *M*) and FAD (0.1 ml. of 10^{-4} *M*) added after exposure.

DPN-treated series is used as a control for a series having both ultraviolet and DPN, the DPN-treated suspensions show greater inactivation than untreated. FAD has a more pronounced inhibitory effect. This may well result from the competition between the normal electron transport pathway in the mitochondrion and the FAD for excess electrons, when the system is partially damaged. Similarly, the system was not reactivated by light under our conditions. Treatment with visible light increased the damage in most cases even when filtered to remove far red and ultraviolet components. No attempt was made to use a narrow region of the visible near 3600 Å in these studies; however, the treatment used is similar to that used to reactivate bacteria successfully. Addition of cytochrome-*c* to the suspension did not result in reactivation by visible light.

Table III presents the data obtained in studies of the effect of infrared radiation on phosphorylation. It is important to note that this process is temperature-sensitive and that any rise in temperature during the infrared treatment results in inactivation of the phosphorylation mechanism. For this reason, the data reported here are those from studies in which the temperature was carefully controlled and kept below 10° C. Under these conditions, there is no effect of infrared treatment either alone or in combination with ultraviolet or gamma radiation. This statement must have one reservation, however; the variability between runs was rather high in these experiments as a result of the extended time of treatment and the dilution necessary for gamma radiation. As a result the averages may not present a completely accurate picture. In the case of pretreatment with infrared and treatment with ultraviolet radiation, three runs showed some increased inactivation and two showed decreased inactivation. There is a suggestion in these data that the infrared may have a slight effect on the phosphorylating mechanism, but nothing of the order of magnitude of its other known effects.

TABLE III

The effect of infrared radiation alone and in combination

Treatment*	No. runs	Phosphate uptake (μM)	% Decrease	% Decrease O_2
Control	3	13.8 ± 1.7	—	—
3 hrs. IR	3	14.0 ± 2.1	− 1.4	−2.2
20 min. UV	5	10.5 ± 1.5	23.9	4.2
3 hrs. IR + 20 min. UV	5	10.3 ± 2.0	25.4	5.1
Control	3	12.8 ± 1.5	—	—
20 min. UV	3	8.8 ± 1.0	31.2	6.7
20 min. UV + 3 hrs. IR	3	8.8 ± 1.0	31.2	5.9
Control	7	9.9 ± 2.5	—	—
2×10^4 r γ	10	5.4 ± 0.8	45.5	1.5
2×10^4 r γ + 3 hrs. IR	10	5.2 ± 0.8	47.5	−1.4

* Particulates undiluted for ultraviolet and infrared treatment; ultraviolet exposure in a rotating quartz flask; infrared exposure in a rotating 5-ml. test tube. Particulates diluted 1 in 20 for γ ray exposure.

In Table IV are presented the data from studies of the effect of combined ultraviolet and gamma radiation. The figures in this table need explanation. The wide discrepancy between the two types of experiment with regard to the inactivation by ultraviolet alone is the result of the fact that to treat the mitochondria with gamma radiation the mitochondria must be diluted. Consequently in studies of the effects of post-treatment with ultraviolet the mitochondria had been diluted, centrifuged and re-suspended before treatment with ultraviolet. In this condition they seem more sensitive. Because of the difficulties of doing studies of this kind, these data can only be taken as *suggestive*. It would appear that the gamma radiation sensitizes the phosphorylation mechanism to ultraviolet treatment, but not the converse. However, unless these studies can be carried out in some manner which is not so drastic to the mitochondria, it will be impossible to be sure of this point. Attempts are being made to do this at present.

The oxidative phosphorylation of isolated particulates is sensitive to both aging and dilution of the preparation. However, in undiluted preparations kept in the cold (below 10° C.), the aging effects are slight for the first 4 or 5 hours. Therefore, most experiments reported in this paper were conducted under conditions in which the mitochondria were in fresh condition at the time of assay. Even the delay between isolation and assay of three hours, experienced with preparations treated with infrared radiation, caused no appreciable decrease in phosphorylative ability due to aging. On the other hand, when a preparation is diluted, the phosphorylative ability is damaged. Therefore, the experiments done with gamma radiation show the superimposition of two effects: dilution and exposure to radiation. The degree of inhibition resulting from dilution varies from preparation to preparation. We are unable to explain this at the present time. This double effect in the gamma-treated series may be obscuring an effect that the radiation would have on undiluted preparations. The part of oxidative phosphorylation which is sensitive to dilution may be highly sensitive to radiation. However, this only means that the effects reported are the minimum effects of radiation. It should be emphasized that the effect of which we speak is a decrease in the total phosphate uptake and not a serious change in the P:O ratio. Some uncoupling occurs in highly diluted preparations but none occurs in undiluted preparations or in diluted preparations which are not allowed to stand in the diluted condition.

TABLE IV

The effect of combined ultraviolet and gamma radiation

Treatment*	No. runs	Phosphate uptake (μM)	% Decrease	% Decrease O_2
Control	3	11.8 ± 1.4	—	—
5 min. UV	3	9.2 ± 1.5	22.0	3.4
10^4 r γ rays	3	11.2 ± 1.0	5.0	− 5.6
5 min. UV + 10^4 r γ rays	3	9.2 ± 0.03	22.0	5.1
Control	3	8.7 ± 2.1	—	—
5 min. UV	3	3.3 ± 0.1	62.1	26.9
10^4 r γ rays	3	4.1 ± 0.9	52.9	22.4
10^4 r γ rays + 5 min. UV	3	2.0 ± 1.2	77.0	28.2

* Particulates undiluted for ultraviolet treatment; diluted 1 in 20 for γ ray treatment.

All the data presented in these tables represent the average values for the experiments, with standard errors. The problem of the variation from run to run cannot be eliminated at this time. The mitochondria for each run are obtained from a different rat. Phosphate uptake and sensitivity to radiation varies from rat to rat. Attempts to standardize the procedures by using rats of uniform age and of defined diet reduce this variation only slightly. Therefore, all of these data must be regarded as indicating relationships in a manner that is qualitative rather than quantitative.

DISCUSSION

The data presented in Table I show quite clearly that ultraviolet radiation in the wave-length region of 2400–2700 Å (probably 2600 Å) readily inactivates the oxidative phosphorylation mechanism of isolated cellular particulates. Furthermore, it is clear that the inactivation of phosphorylation is unrelated to inactivation of the oxidase system, since oxygen uptake may be very slightly affected in some cases in which phosphorylation is quite low. The separation of phosphorylation from oxidation is a common observation in mitochondrial preparations. Aging of the preparation will cause a loss of phosphorylative ability without appreciable damage to the oxidative system. Ultraviolet radiation merely accelerates this process. The observations with ultraviolet radiation are in complete agreement with those using gamma radiation (Yost and Robson, 1957). It appears that any one of a variety of agents will uncouple phosphorylation and oxidation.

The use of ultraviolet radiation to uncouple the phosphorylation mechanism presents some interesting approaches to the general problem of radiation effects and to the general problem of the sequence of events in oxidative phosphorylation. On this last point, Beyer (1958) has recently suggested that reversal of the effects of ultraviolet radiation may be used as a tool to indicate the normal pathway of electron flow in this system. His observation that addition of vitamin K is necessary to reactivate the glutamate system may be taken as evidence that this vitamin participates in the over-all process. Similar experiments may be done to elucidate further the mechanisms of phosphorylation. However, the observations of Beyer that photoreactivation can be achieved after treatment with ultraviolet radiation are not substantiated by the data presented in this paper. Under the conditions employed in these studies, it has been impossible to reactivate phosphorylation with visible light or the addition of several co-factors. Although the inactivation of oxidative phosphorylation by ultraviolet radiation may play a large part in the death of cells exposed to radiations, and although it is a well known fact that cells exposed to lethal doses of ultraviolet radiation can be revived by subsequent exposure to visible radiation (Kelner, 1949), the results reported in Table I indicate that the isolated particulate system cannot be reactivated by the same means that the whole cells can. Whether this inability to reactivate the phosphorylation mechanism is the result of a failure to use the right wave-lengths (3600 Å) exclusively, or is the result of failure to combine co-factor addition with light treatment can only be determined in the future. Whether the phosphorylation mechanism is inactivated in whole cells by ultraviolet radiation and then revived by light treatments will be the subject of a new series of investigations.

Since the report of inactivation and photoreactivation of DPN (Wells, 1956), it has seemed possible that any interference with oxidative metabolism might be the result of inactivation of the nucleotide-containing co-enzymes. These were tried in this system in attempts to reactivate phosphorylation although they have no known function in the succinate system. That they failed to reactivate the system is not surprising. It does indicate that the decrease in phosphorylative ability is not the result of inactivation of some other (nucleotide-requiring) part of the system. Since ATP is a normal part of the reaction mixture, it is safe to assume that the effect is not upon the adenosine nucleotides. This agrees with the findings of Beyer (1958).

The data in Tables III and IV represent an attempt to find a mechanism of action for combined radiation effects. The effects of combined radiations on chromosomes must have their explanation either in the alteration of the ability of the breaks to rejoin or in the alteration of the fragility of the chromosome to radiation. Unless a mechanism of action can be discovered, it is unlikely that any satisfactory explanation will be forthcoming. The many experiments indicating that the damage to chromosomes by radiation is greatly influenced by known inhibitors of phosphorylation (Wolff and Luippold, 1955) suggests that studies on the combined effects of radiations on phosphorylation might give some important clues to radiation mechanisms. The data gathered in these experiments indicate that there is no pronounced effect of combined treatments with ultraviolet, infrared and gamma radiations. The potentiating effect of infrared, so clearly demonstrated in Tradescantia (Swanson, 1949), cannot be found in the mitochondrial systems; nor is there any indication of interactions (other than an additive effect of the radiations) between gamma rays and ultraviolet. These findings are not in accord with the report by Gordon and Surrey (1958) that near-infrared radiation accelerates the decay of phosphorylative ability in rat liver mitochondria. The techniques employed in the two studies are quite different and the difference in results is undoubtedly attributable to the greater sensitivity of the technique of Gordon and Surrey. Indeed this may explain the variability of some of our results. It is possible that a slight infrared effect is present in our test materials, but that our test is too insensitive to give a consistent measure of its magnitude. However, there can be little comparison of an effect on phosphorylation of this small degree with the enormous effect of infrared radiation on chromosome breakage. It seems highly unlikely that an increase in x-ray breakage of chromosomes amounting to 100 to 200 per cent can be induced by alterations of the phosphorylation mechanism too slight to be detected by the hexokinase method employed in this study.

SUMMARY

Data are presented which show that ultraviolet radiation in the 2600 Å region inactivates oxidative phosphorylation. The addition of co-factors and the exposure to light of the preparations failed to reactivate the phosphorylation after treatment with ultraviolet. Treatment with infrared radiation and treatment with combined infrared and ultraviolet, infrared and gamma, and ultraviolet and gamma radiation failed to show any alteration in the normal inactivation patterns found with treatment with ultraviolet or gamma radiation alone. These data suggest that both ionizing and non-ionizing ultraviolet radiations damage cells, at least in part, by inactivation of oxidative phosphorylation. However, these data do not give any clue to the mechanism of action of combined radiations in altering the chromosomal structure of cells.

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